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The Relation between Variability and Abundance  
shown by the Measurements of the Eggs of  
British Nesting Buds

BY R. A. FISHER, F.R.S.

(Received 9 May 1936)

I. ORIGINS OF RESEARCH

For some years it has appeared to the author of importance to ascertain by collocation of direct observations whether, in fact, Darwin's generalization that "widely distributed, much-diffused and common species vary most", is well established, and, if so, whether we can form an idea of the quantitative relationship between variability and abundance. In a paper published nine years ago in collaboration with Mr E. B. Ford (1928), I examined the variability, shown by Mr Ford's observations, of thirty-five species of night-flying moths, mostly British, from which examination it appeared that the difficulties in the way of comparing variabilities could be overcome at the expense of some elaboration of the calculations. When a satisfactory basis of comparison had been obtained it appeared that in both sexes the group of species classified in entomological handbooks as "abundant" or "very common" were, in fact, on the average, some 60% more variable than the group of species classified as less than common, while the group of species classified as common held, in both sexes, an intermediate position. Within each of these three groups there was a wide range of variability and I should give an altogether false impression if I suggested that variability among these thirty-five species was closely associated with abundance, or that, if influenced by abundance, it is not also much influenced by other causes. Nevertheless, the apparent influence of abundance was, in this research, statistically significant, and encouraged me to think that other cases might be found in which the reality of the phenomenon could be tested and its numerical importance gauged.

For many years the Rev. F. C. R. Jourdain has made and compiled measurements of considerable numbers of the eggs of British nesting birds. At the suggestion of Mr Julian Huxley, I approached Mr Jourdain, proposing at the same time that, in order to ensure the preservation of the large number of individual measurements he had made, I could have them copied in my

department, in a form suitable for permanent preservation in the Archives, which the British Museum (Natural History) had established for the reception of bodies of original biological measurements. Mr Jourdain very kindly let me have his measurement sheets from time to time, and, over a period of 5 years, the primary reduction of each set of 200 measurements was carried out, species by species. It is the result of this undertaking that I wish here to put on record. I desire that it should be understood that I myself took no part in the collection or measurement, and could have undertaken no part of the work had not Mr Jourdain's measurements been generously placed at my disposal. I trust that the original data will eventually be made public, by being deposited at South Kensington. For my own enquiry is not the only one that might be based upon them, and it may be that I should have done better if, in some respects, I had treated the data somewhat differently.

Measurements of eggs have been used solely because they are available, and the data have the particular value that they allow a large number of species to be compared, each species being represented by a fairly considerable sample. The relative size of different individuals would certainly be better represented by measurements of representative bones, or by the total weight of the body. In undertaking the research I felt no assurance that the measurements of the eggs did not vary so greatly from causes unconnected with the size of the mother as to make them useless as a gauge of variability in the different species. Had that been the case, I should merely have failed to establish any association between variability and other factors. It was a ground for hesitating to undertake the enquiry, not for hesitating to accept any results to which it might lead.

Had the data been collected for the object I had in view, it would have been preferable to measure 100 eggs of different clutches, rather than a total of 100 eggs from a variable number of clutches which were much larger, for example, among the game birds than among the pigeons. On trial I find that measurements of eggs belonging to the same clutch very generally vary less than do eggs from different clutches, so that, had it been necessary to try to compare the variabilities of species of different orders, the comparison would have been appreciably more precise if only one egg had come from each nest. The available data, however, had not been collected in this way, and the varying size of the clutch thus provided only one more reason for restricting the comparisons within groups of related birds. I have not thought it necessary to apply the refinement of eliminating the variability within clutches, but have taken the series of 100 measurements available as representing each species. The similarity of the measurements of eggs

of the same clutch, while it has been thus a minor technical embarrassment, is satisfactory evidence that measurements of the eggs are, in a real sense, indirect measurements of their mothers.

## 2—THE EQUILIBRIUM OF MUTATION AND SELECTION

The theoretical reasons for anticipating a higher variability associated with a numerically larger population are recondite and by no means fully understood. They depend on the balance of selective and mutational agencies, by which the genetic variability of natural populations must be determined. It is because we cannot easily predict these *a priori* that direct empirical knowledge is of importance. In respect of variations conditioned environmentally, there is no reason to suppose that these would be greater or less in a dense than in a sparse population. The same is true of that fraction of the variability which is caused by deleterious mutations. To these all species appear to be exposed. It is important to note that these are the only mutations which can have an appreciable mutation rate. Some of them, in *Drosophila* and maize, have mutation rates of the order of one in a million in each generation, and a few are even more frequent. But no beneficial mutation, even if the benefit it conferred were almost infinitesimally slight, can have a mutation rate of this order, else it would long ago have established itself throughout the species concerned, and so would no longer appear as a mutation. We are therefore left to consider only "neutral" mutations, together with the beneficial mutations which must be exceedingly infrequent. In saying this I do not refer to the aggregate frequency, for the different kinds of mutations possible may be exceedingly numerous. Each of these kinds, however, must be so infrequent in its occurrence that, in the entire species, each occurs no more frequently, perhaps, than once in a thousand generations.

A perfectly neutral mutation is, of course, a mathematical fiction, like a perfect equipoise between the weights on the scales of a balance. Moreover it has been shown (Fisher 1930) that to be effectively neutral the equipoise between the selective advantages of the contrasted genes must be almost incredibly exact. It is difficult, therefore, to claim for this class of mutations any real importance. Its importance for us rests on the fact that, like other mathematical fictions, it affords a convenient basis for calculation.

If species of different populations were supplied at the same absolute rate with different neutral mutations, each occurring with extreme rarity, then calculation shows that the number of factors maintained in the



heritable variance of the species would be nearly proportional to the logarithm of the population. For example, if one appeared in each generation, this would be enough in a population of a million to maintain, on the average, 30.4 factors segregating. While in a population of a billion (a million million) it would be enough to maintain 58.0. The additional segregating factors in the more numerous species would, indeed, add little to its observable variance because of the very great inequality of the gene ratios with which, in such species, factors can be maintained. They would, on the other hand, be available to increase the observable variance whenever, by change of circumstances, the mutant gene happened to become slightly advantageous. When this occurs the gene ratio will shift slowly towards equality, and the contribution of the factor in question to the observable variability of the species will steadily increase, until the rival genes are equal in numbers. After this it will decrease again as the new gene replaces the old. Such a course of events must often take thousands of generations. It appears that only if the more numerous species, through its capacity for keeping a larger number of neutral mutations in store for future use, or because of the larger number of individuals exposed to the chance of very rare advantageous mutations, manages to maintain a larger supply of new genes, each gradually spreading throughout the whole population, that its observable variance will be larger than that of a species of smaller population. Hence, although I believe many unknown factors may largely affect the variation of different species, any consistent difference in variability, observable between more and less populous species, must be ascribed to that fraction of the specific variance contributed by those particular factors by means of which evolutionary progress is now taking place. It is for this reason that I believe the attempt to ascertain whether such a difference in variability can be measured is worth the very considerable computational labour which the attempt involves. It would be too much to claim that we have here a means of measuring the rate of evolutionary progress. What it does supply is the order of magnitude of the contribution to the observable variance, of those genetic factors by means of which evolutionary progress is now proceeding.

### 3—THE CALCULATION AND ADJUSTMENT OF VARIABILITY

Table I shows for each of 180 populations of birds the five statistics calculated from the measured lengths and breadths of, in nearly all cases, 100 eggs. If  $x$  and  $y$  stand for the length and breadth of any egg, these five statistics are the following:

- (i) The mean length and mean breadth calculated from the formulae

$$\bar{x} = \frac{1}{n} S(x),$$

$$\bar{y} = \frac{1}{n} S(y),$$

where  $n$  stands for the number of eggs measured and  $S$  for summation over the observed values

- (ii) The mean squares and product of deviations from the means, namely the variance of length

$$\frac{1}{n-1} S(x-\bar{x})^2,$$

the variance of breadth  $\frac{1}{n-1} S(y-\bar{y})^2,$

and the covariance of length and breadth

$$\frac{1}{n-1} S(x-\bar{x})(y-\bar{y})$$

The first four of these statistics are always positive, but the covariance, though in fact it is generally positive, may be of either sign. For example, among the thirteen gulls in the list, the covariance is positive in all cases save the Arctic skua (*Stercorarius parasiticus*) for which it has a small negative value.

An examination of any group such as the gulls displays the first difficulty encountered in making any valid comparison of variability. The smallest bird of the group, the lesser tern (*Sterna albfrons*) has a variance in length just under 2 mm<sup>2</sup>, and in breadth a little more than 0.5 mm<sup>2</sup>, while the largest of the group, the greater black backed gull (*Larus marinus*) gives 7.4 mm<sup>2</sup> for length and 2.0 mm<sup>2</sup> for breadth. Both values are nearly four times as great as those for the smaller bird. Obviously, in both cases the variance is much greater chiefly because the eggs are much larger. The mean length for the black backed gull 77 mm, is considerably more than twice that for the lesser tern, 33 mm, while the breadth also is greater in nearly the same proportion. It is clear that no valid comparison of variabilities can be made unless a method is found of making allowance for the average length and breadth observed in each species. Owing to the wide range in size in almost all the groups, these allowances will have to be considerable in magnitude, and the validity of the comparisons to be made must depend entirely on this process having been properly carried out, both as to principle and as to accuracy.

TABLE I—PRIMARY STATISTICS AND STANDARDIZED VARIANCES OF EGG LENGTH AND EGG BREADTH FOR 180 POPULATIONS OF BRITISH NESTING BIRDS

	No of eggs	Mean length mm	Mean breadth mm	Variances		Co variance mm <sup>2</sup>	Standardized variances	
				Length mm <sup>2</sup>	Breadth mm <sup>2</sup>		Length	Breadth
<b>Accipitres</b>								
<i>Aquila chrysaetos</i> L	100	77.020	59.485	15.708	6.9698	4.5164	83.81	125.55
<i>Haliaeetus albicollis</i> L	54	75.809	58.709	16.401	7.8514	9.5308	87.58	130.76
<i>Buteo buteo</i>	100	56.798	45.445	4.8317	2.6469	2.8689	67.54	107.50
<i>Nitidus nitidus</i>	58	56.978	45.090	6.8814	1.8381	1.6926	81.51	92.50
<i>Falco peregrinus</i> T	100	51.803	41.001	3.7375	1.6007	0.61182	65.12	95.09
<i>Fernis opimus</i> L	23	50.383	41.717	3.2206	1.5224	1.1442	65.32	90.89
<i>Circus cyaneus</i> L	107	46.305	36.077	6.4410	2.9039	1.4445	99.26	116.60
<i>Falco subbuteo</i> L	100	41.573	32.732	2.1186	1.2276	0.40279	63.15	104.01
<i>Accipiter nius</i> L	100	39.827	31.836	4.7697	2.3955	2.5186	104.14	135.41
<i>Falco tinnunculus</i> L	100	39.737	31.771	2.2015	0.95622	0.64604	72.75	95.72
<i>Falco columbarius</i>	100	39.950	31.267	1.5714	0.85244	0.45833	53.87	93.88
<b>Alcae</b>								
<i>Uria ualge</i>	100	81.726	50.063	8.4545	3.4502	1.5346	31.44	112.75
<i>Uria ualge albionis</i> W	100	81.466	49.671	12.865	3.2526	2.4427	49.63	111.20
<i>Alca torda</i> L	100	73.080	46.859	9.7063	2.9012	2.1057	53.05	110.92
<i>Fratercula arctica</i> L	100	60.802	42.297	4.3010	1.4227	0.02890	43.72	88.48
<i>Uria grylle</i> L	102	58.243	39.743	3.7772	1.3641	0.06939	41.02	92.48
<b>Anseres</b>								
<i>Anser anser</i> L	100	85.319	58.040	15.764	4.9455	3.9656	62.73	114.16
<i>Sonasteria mollissima</i> L	100	77.145	50.712	10.812	1.9449	1.9004	54.05	86.22
<i>Tadorna tadorna</i> L	100	68.813	47.584	3.2922	1.5453	0.14374	27.02	74.96
<i>Meergus merganser</i> L	100	66.383	48.409	2.7051	1.2447	0.54490	14.74	74.23
<i>Meergus serrator</i> L	100	65.559	45.120	4.3424	1.2273	-0.01150	35.35	76.31
<i>Oedemia nigra</i> L	72	65.226	44.849	5.5580	1.4361	0.36474	46.50	83.72
<i>Nyroca ferina</i>	100	60.734	44.000	3.9004	1.1008	0.28869	42.97	73.34
<i>Anas platyrhynchos</i> L	100	57.177	41.031	2.4028	1.5405	1.0713	42.57	94.38
<i>Nyroca fuligula</i> L	100	57.787	40.595	3.1848	1.0338	-0.00280	36.68	78.41
<i>Anas strepera</i> L	100	54.268	39.093	2.3119	1.8858	0.06709	31.55	107.58
<i>Anas penelope</i> L	100	53.930	38.253	4.2631	2.0191	0.90567	57.55	112.58
<i>Anas acuta</i>	100	54.199	37.440	3.8470	2.1984	0.89091	50.34	118.50
<i>Spatula clypeata</i> L	100	51.996	37.172	4.6959	0.85557	1.1913	66.20	77.82
<i>Anas crecca</i>	100	45.478	33.449	2.8815	0.90778	0.69200	61.33	89.64

[illegible]

TABLE I—(continued)

	No. of eggs	Mean length mm.	Mean breadth mm.	Variances			Co-variances		Standardized variances	
				Length	Breadth	mm. <sup>2</sup>	mm. <sup>2</sup>	mm. <sup>2</sup>	Length	Breadth
Laniidae										
<i>Numericus arcticus</i>	100	67.610	47.910	11.437	3.1109		2.3469		76.57	111.00
<i>Numericus phaeopus</i>	85	58.485	41.706	4.3068	1.8650		0.59580		48.91	101.19
<i>Oxyechus oestrupus</i>	101	57.008	39.776	8.3329	2.5174		-0.31591		79.46	118.81
<i>Burhinus oedemus</i>	100	53.788	38.436	9.2437	1.4850		-0.39391		92.02	98.74
<i>Charadrius aprinarius</i>	100	51.770	35.880	2.9360	1.0310		0.19000		43.71	89.42
<i>Tringa nebularia</i>	100	51.434	34.811	5.0680	1.0510		0.66200		66.10	93.25
<i>Vanelius vanellus</i>	100	47.090	33.710	5.0760	1.4250		-0.31600		80.14	109.00
<i>Scolopax rusticula</i>	100	44.199	33.528	2.8865	0.71372		0.32298		67.07	78.67
<i>Tringa totanus</i>	100	45.180	31.660	1.7930	0.68500		0.31100		37.26	93.49
<i>Charadrius normellus</i>	100	41.102	28.872	3.4911	0.65517		0.37844		76.57	89.27
<i>Capella gallinago</i>	104	39.338	28.636	2.8690	1.1002		0.45483		75.62	112.17
<i>Tringa hypoleuca</i>	100	36.418	26.277	2.0486	0.36058		0.07012		68.37	71.58
<i>Charadrius hiaticula</i>	100	35.750	25.960	2.1430	0.51800		0.52300		72.77	88.35
<i>Calidris alpina</i>	100	34.770	24.770	1.8600	0.35600		0.24700		67.96	76.51
<i>Charadrius alexandrinus</i>	100	33.049	23.498	0.94800	0.30600		0.18600		43.85	74.74
<i>Phalaropus lobatus</i>	100	29.957	21.000	1.2348	0.25434		0.19141		64.48	77.02
Poaceae										
<i>Accentornis</i>										
<i>Alcedor modularis</i>	100	19.888	14.725	0.57682	0.24533		0.085152		79.98	107.02
Alaudidae										
<i>Alauda arvensis</i> L.	100	23.770	17.057	0.92232	0.40369		0.028798		78.06	115.46
<i>Lullula arborum</i>	101	21.612	16.348	0.61986	0.22732		0.075830		75.15	94.02
Cathartidae										
<i>Cathartes familiaris</i> L.	100	15.519	12.092	0.37059	0.077898		0.002139		90.03	74.51
Cuculidae										
<i>Cuculus cinclus</i>	100	26.181	18.614	2.2276	0.59776		0.46956		105.41	124.91
<i>Cuculus cinclus</i>	100	25.774	18.668	1.5064	0.46105		0.57832		94.41	113.17
Corvidae										
<i>Corvus corax</i> L.	100	49.721	33.401	9.2116	2.2981		2.1536		95.04	131.04
<i>Corvus cornix</i> L.	100	43.660	30.360	5.7959	0.83420		0.54910		91.84	95.30
<i>Corvus corone</i> L.	100	43.260	30.510	5.4377	0.78430		0.43970		90.77	90.94
<i>Corvus frugilegus</i> L.	100	40.040	28.330	6.7420	0.75260		0.38310		108.60	96.94
<i>Pyrrhocorax pyrrhocorax</i>	100	39.410	27.940	1.4032	0.83590		0.00470		42.17	102.74
<i>Colinus ocellatus</i>	100	35.728	25.498	2.2774	0.75959		0.44894		74.05	106.79
<i>Ficedula hypoleuca</i>	100	34.130	24.270	6.4484	0.87020		0.50120		123.77	117.18
<i>Garrulus glandarius</i>	100	31.740	22.850	1.7130	0.64150		0.34200		74.85	109.28



TABLE I—(continued)

	No of eggs	Mean length mm.	Mean breadth mm.	Variances		Co-variance mm. <sup>2</sup>	Standardized variances	
				Length mm. <sup>2</sup>	Breadth mm. <sup>2</sup>		Length	Breadth
<b>Paridae</b>								
<i>Parus major</i>	100	17.986	13.624	0.45702	0.20306	0.10494	81.33	105.62
<i>Parus cristatus scoticus</i>	100	16.273	12.742	0.24502	0.12367	0.07812	67.48	89.81
<i>Parus palustris</i>	100	15.793	12.308	0.42732	0.10680	0.07026	94.41	86.63
<i>Parus coerulesus</i>	100	15.444	11.891	0.44533	0.098049	0.042542	97.57	86.15
<i>Parus ater</i>	100	15.004	11.631	0.32120	0.10903	0.02250	86.96	92.89
<i>Agredalobus caudatus</i>	100	14.171	11.004	0.27238	0.081196	0.011936	85.95	84.87
<b>Regulidae</b>								
<i>Regulus regulus</i>	100	13.605	10.220	0.30694	0.088081	-0.019091	92.67	96.44
<b>Sittidae</b>								
<i>Sitta europaea</i>	100	19.199	14.323	0.87586	0.22644	0.15871	101.43	105.99
<b>Sturnidae</b>								
<i>Sturnus vulgaris</i>	100	30.230	21.250	2.4324	0.24470	0.13890	93.23	74.23
<i>Sturnus vulgaris</i>	52	31.133	21.992	1.8587	0.82935	0.19529	78.85	124.08
<b>Sylvinae</b>								
<i>Sylvia borin</i>	100	20.130	14.780	0.68400	0.23160	0.04080	84.50	104.19
<i>Sylvia atricapilla</i>	100	19.590	14.740	0.47310	0.26060	0.14930	73.31	108.39
<i>Acrocephalus palustris</i>	100	18.970	14.150	0.70810	0.23480	0.24770	93.46	108.64
<i>Sylvia communis</i>	100	18.590	13.990	0.96790	0.22280	0.26350	108.88	107.32
<i>Acrocephalus scirpaceus</i>	100	18.368	13.639	0.61620	0.16543	0.14408	90.55	101.77
<i>Locustella naevia</i>	100	18.087	13.803	0.94397	0.19201	0.16276	112.85	101.92
<i>Acrocephalus schoenobaenus</i>	100	17.730	13.300	0.71160	0.09620	0.04820	101.27	75.44
<i>Sylvia undata</i>	101	17.527	13.172	0.40458	0.13982	0.03105	78.10	92.54
<i>Sylvia curruca</i>	100	17.270	12.835	0.67990	0.19624	0.10328	101.19	108.80
<i>Phylloscopus collybita</i>	100	16.113	12.622	0.57387	0.18699	0.13951	105.52	108.61
<i>Phylloscopus trochilus</i> L.	100	15.414	12.290	0.33091	0.10879	0.05893	87.77	87.30
<i>Phylloscopus collybita</i>	100	15.350	12.154	0.49040	0.12332	0.03950	104.70	93.84
<b>Troglodytidae</b>								
<i>Troglodytes troglodytes</i>	100	16.658	12.772	0.34105	0.15840	0.07952	77.68	100.55
<i>Troglodytes troglodytes</i>	78	18.508	13.989	0.40176	0.16060	0.14284	72.41	93.19
<i>Troglodytes troglodytes</i>	25	18.588	13.388	0.52943	0.31943	0.04610	80.08	127.40





One obvious method of adjustment would be to divide each variance by the square of the corresponding mean, or in other words, to use the so called coefficient of variation, or its square, the relative variance, as a basis for comparison. The objection I feel to any procedure of this kind is that it is arbitrary, that is to say that it depends on the choice of the investigator. If his choice be wrong he will make too great or too small an allowance for the size of the egg, and, in consequence, will make the smaller birds of each group appear more or less variable than they ought in comparison with the larger birds. For a comparison with abundance any such error would be fatal. Abundance is not likely to be independent of the size of the bird. In fact, in many groups the smaller species must be considerably more numerous in individuals than the larger species. We should thus, by making any such arbitrary choice, be liable to enhance or to diminish the effect we are seeking to detect, largely, and by an unknown amount.

The only adequate solution, as it seems to me is to eliminate entirely any portion of the association between variability and abundance that may be due to the association of both these variables with the average length and average breadth of the eggs. It may be that, if some absolutely correct method of allowance for size could be imagined, large eggs would be found to be more or less variable than small eggs of related species. If so, this fact would, I hold, only be relevant to our enquiry as a disturbing factor, which we should wish to eliminate in studying whether, apart from size, variability is associated with abundance.

If this point of view is correct the allowance we must make for the average length and breadth of the eggs of each species must be that provided by the data themselves in such a way that, after allowance has been made, neither the larger nor the smaller birds of each group are on the whole the more variable. I have, therefore, first replaced the means and variances by their common logarithms, and then calculated, from the sums of squares and products within groups the actual regressions of the logarithm of variance in length, the logarithm of variance in breadth, regarded as two dependent variates, on the logarithm of the mean length, and the logarithm of the mean breadth, as independent variates. If  $L$  and  $B$  stand for the logarithms of the mean lengths and breadths, and  $u$  and  $v$  stand for the logarithms of the variances of length and breadth, the allowances so found empirically from the data provided by 137 independent comparisons within groups are

$$U = 4.327258L - 1.909220B,$$

$$V = -0.236169L + 2.320619B$$

where  $U$ ,  $V$  may be regarded as corrections to be subtracted from  $u$ ,  $v$  in order to render them comparable. The exact procedure is shown in the Appendix on the Analysis of Covariance. These allowances differ greatly from what would have been used had the coefficient of variation been adopted. This would have been equivalent to using the formulae

$$U = 2L + 0B,$$

$$V = 0L + 2B,$$

and the comparison shows that the facts of nature are very different from those supposed in these last equations. An increase of size without change of shape would entail an equal increase in  $L$  and  $B$ . In the theoretical formulae this produces an increase twice as great both in  $U$  and  $V$ . In fact it is followed, as the two formulae show, by a relative increase of  $2.418 \pm 0.177$  in  $U$  and  $2.084$  in  $V$ . Both values show a higher allowance than that supplied by the coefficient of variation, though in the second case the deviation is not statistically significant. A change in size, even without change in shape, would not be adequately represented by the coefficient of variation.

The agreement is still more unsatisfactory if we consider a change in shape without change of volume, such as would be produced by decreasing  $B$  by one unit, and increasing  $L$  by two. The theoretical change in  $U$  is then 4. But the observed coefficient is 10.544, with a standard error 3.538. For  $V$  the deviation is less, but in the opposite direction, for instead of  $-2$  we obtain  $-2.793$ , with a standard error 4.209. The difference  $V - U$  is increased by 13.357, with a standard error 2.946, whereas the theoretical increase is only 6. It appears therefore that, with increase of size within groups of related birds, variability in length at least increases more rapidly than if the coefficient of variation were constant, and that with change of shape, e.g. broadening and corresponding shortening, the relative variability of breadth to that of length increases still more rapidly.

The method of adjustment adopted has been designed for use in comparisons within groups, and has been obtained from the mean squares and products within these groups only, ignoring all differences from one group to another. The corrected values may not be thought therefore, ideal for making comparisons of group with group, yet it is not without interest to see how the averages of the adjusted variances of the different groups compare with one another. In making these and other comparisons it will be convenient to multiply the numerical values of  $u - U$  and  $v - V$  by 100 to obtain a more convenient unit of comparison. Since common logarithms

to the base 10 were used, this new unit is equivalent to a difference of 2.3026 % ( $\log_e 10$ ).

The average values for adjusted variances in length and breadth are shown simultaneously in fig. 1. Families or subfamilies of passerine birds are marked with a cross, the non-passerine orders being shown by circles. The number of species averaged in each group is indicated

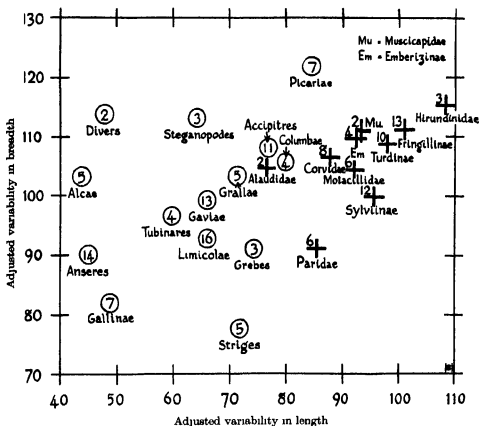


FIG. 1.—Mean corrected variance for length shown from left to right, and for breadth upwards. Non-passerine groups are shown by circles, and passerine groups by crosses. The numbers indicate the number of species averaged in each group. The positions of the smaller groups have little accuracy. Each unit represents 2.3026 %

One very obvious fact brought out by this diagram is the generally greater variability of the passerine species. If we judge by the variance figure for length, only the two larks, the least variable of the passerine groups, fall among the non-passerine orders, while among these the picarian

birds stand out as exceptionally variable. For the latter I have shown the average of seven species, excluding the cuckoo, which, if it had been included, would have further raised the average in both respects. The comparison is, probably, to some extent influenced by size, though it should be noted that the tits and the wren are among the least variable of the passerine birds. It seems not unreasonable to suggest that the difference in variability is connected with more recent evolutionary modification among the passerine groups.

#### 4—COMPARISONS WITHIN GROUPS

The purpose of the adjustments to which the variances have been subjected has been to render them comparable within groups, and in these to make comparisons between species believed to be more and those believed to be less numerous. To obtain estimates of relative abundance which should be entirely uninfluenced by the variability figures with which they were to be compared, I have had recourse to Mr Alexander who has had much experience in the estimation of bird numbers, and in consultation with colleagues in this country and abroad has subdivided all the groups I submitted to him into two, three or four classes, representing abundance in the world population, so far as it can be estimated. In spite of the great care which has been given to this classification, I cannot hope that in every case the species have been arranged in the best order, still less that large differences in real abundance have not, in some cases, passed unrecognized. All that I can claim is to have used the best indications of abundance available and that, where species have been assigned to the wrong abundance classes, this had been done without knowledge of the figures for variability. Misclassification might, therefore, diminish the apparent contrast in variability between more and less abundant species. Indeed, if misclassification is frequent it must have led to a serious under estimate of this contrast. It could not, however, possibly have produced any consistent difference in the average variabilities of the species judged to be more or less abundant.

Table II gives the groups classified in only two abundance classes. These are the five gullmotes and two divers among non passerine birds, two larks, two flycatchers, six wagtails and pipits, and three martins among passerine birds. Of these twenty species, ten fall into the class judged to be rarer and ten into the class judged to be commoner than other members of their group.

The table shows for each species deviations from the mean values for each group, of the adjusted variances in length and breadth. The average

TABLE II—COMPARISON OF DEVIATIONS FROM GROUP MEANS OF ADJUSTED VARIABILITIES IN LENGTH AND BREADTH OF EGG, FOR GROUPS DIVISIBLE IN TWO ABUNDANCE CLASSES

	Rarer	Variability in		Commoner	Variability in	
		Length	Breadth		Length	Breadth
<b>Alcae</b>						
Razorbill		9 28	7 75	Southern guillemot	5 86	8 03
Black guillemot		- 2 75	- 10 69	Northern guillemot	- 12 33	9 58
				Puffin	- 0 05	- 14 69
<b>2 Alcae</b>		<b>6 53</b>	<b>- 2 94</b>	<b>3 Alcae</b>	<b>- 6 52</b>	<b>2 92</b>
<b>Alaudidae</b>						
1 Wood lark		- 1 45	- 10 82	1 Skylark	1 45	10 82
<b>Muscicapidae</b>						
1 Pied flycatcher		- 2 48	9 45	1 Spotted flycatcher	2 49	- 9 45
<b>Motacillidae</b>						
Grey wagtail		2 52	1 06	Meadow pipit	- 20 91	- 9 04
Water pipit		- 0 24	20 65	White wagtail	9 06	10 55
Tree pipit		- 1 64	- 23 29	Yellow wagtail	11 20	0 06
<b>3 Motacillidae</b>		<b>0 64</b>	<b>- 1 58</b>	<b>3 Motacillidae</b>	<b>- 0 65</b>	<b>1 57</b>
<b>Hirundinidae</b>						
House martin		- 9 58	5 20	Swallow	9 04	10 46
Sand martin		0 52	- 15 64			
<b>2 Martins</b>		<b>- 9 06</b>	<b>- 10 44</b>	<b>1 Swallow</b>	<b>9 04</b>	<b>10 46</b>
<b>Colymbidae</b>						
1 Black throated diver		- 1 90	4 11	1 Red throated diver	1 89	- 4 10
10 Totals		- 7 72	- 12 22	10	+ 7 70	+ 12 22
Means		- 0 772	- 1 222		+ 0 770	+ 1 222

for the ten commoner species exceeds that for the ten rarer species by 1 542 in length and by 2 444 in breadth. These differences correspond to average differences of about 3.6 and 5.6% in the variances. The differences are small, but both are in the direction indicated by Darwin's law. If only these twenty species were available, the law would only be supported; it could not be said to be demonstrated by the data.

Table III gives the groups for which three abundance classes are available. These are the woodpeckers, owls, doves, cormorants, plovers, game birds, rails, grebes, and petrels, together with the tits, buntings, and finches. Out of seventy-three species, twenty are placed in the rarest of three classes, thirty-two in the middle class and twenty-one among the commonest. The rarest class shows a variability less than the average in both length and breadth. The same is true to a less extent of the middle class, while the commonest class is above the average in both respects. In length the average value of the commonest class exceeds that for the rarest class by

BREADTH OF EGG FOR GROUPS DIVISIBLE IN THREE ABUNDANCE CLASSES

	Variability in		Mid	Variability in		Common	Variability in	
	Length	Breadth		Length	Breadth		Length	Breadth
<b>Rare</b>								
<b>Picraeae</b>								
Green woodpecker	1 57	7 03	Lesser spotted wood pecker	23 84	5 59	Great spotted wood pecker	—	0 17 10 99
			W rymack	— 25 26 — 12 11				
<b>1 Picraean</b>	<b>1 57</b>	<b>7 03</b>	<b>2 Picraeans</b>	<b>— 1 42 — 18 00</b>		<b>1 Picraean</b>	<b>— 0 17 10 99</b>	
<b>Striges</b>			Long eared owl	6 66	5 59	Short-eared owl	— 3 03 — 5 31	
Little owl	2 92	0 86				Barn owl (white)	7 04 5 09	
Tawny owl	— 13 60 — 6 22		<b>1 Owl</b>	<b>6 66</b>	<b>5 59</b>	<b>2 Owls</b>	<b>4 01 — 0 22</b>	
<b>2 Owls</b>	<b>— 10 68 — 5 36</b>		Rock dove	— 9 30 2 31		Turtle dove	1 03 — 6 37	
<b>Columbae</b>			<b>1 Dove</b>	<b>— 9 30 2 31</b>		Wood pigeon	15 30 4 93	
Stock dove	— 7 04 — 0 88		<b>1 Shag</b>	<b>3 73 0 33</b>		<b>2 Doves</b>	<b>16 33 — 1 44</b>	
<b>1 Dove</b>	<b>— 7 04 — 0 88</b>					1 Cormorant	— 2 25 5 41	
<b>Steganopodes</b>								
1 Gannet	— 1 49 — 5 74		Long tailed tit	0 33 — 6 09		Great tit	— 4 29 14 66	
<b>Paridae</b>			Marsh tit	8 79 — 4 33				
Crested tit	— 18 14 — 1 15		Coal tit	1 34 1 73				
			Blue tit	11 96 — 4 81				
<b>1 Tit</b>	<b>— 18 14 — 1 15</b>		<b>4 Tits</b>	<b>22 41 — 13 50</b>		<b>1 Tit</b>	<b>— 4 29 14 66</b>	
<b>Lamprolæ</b>			Whimble	— 17 39 8 49		Redshank	— 29 04 0 79	
Stone curlew	25 72 6 04		Ringed plover	6 47 — 4 35		Curlew	10 27 18 30	
Dotterel	10 27 — 3 43		Red necked phalarope	— 1 82 — 15 68		Sandpiper	2 07 — 21 12	
			Kentish plover	— 22 45 — 17 96		Saupe	9 32 19 47	
			Golden plover	— 22 59 — 3 28		Lapwing	13 84 16 30	
			Greenbank	— 0 20 0 55		Dunlin	1 66 — 16 19	
			Woodcock	0 77 — 14 03				
			Oystercatcher	13 18 26 11				
<b>2 Lamprolæ</b>	<b>35 99 2 61</b>		<b>8 Lamprolæ</b>	<b>— 44 05 — 20 15</b>		<b>6 Lamprolæ</b>	<b>8 12 17 55</b>	



4 613, corresponding to an average difference in variance of 10.6%. In breadth the difference is 14 063, corresponding to 32.4%. Taking the average of the figures for length and breadth, the mean values for the three classes are -3 946, -1 072 and 5 392. The difference between the common birds and the middle class is thus more than twice the difference between the middle class and the rarer birds. Owing to the considerable number of species in this classification the totals and averages seem to possess some regularity.

There are sixty-eight species in the groups classified into four classes, namely the ducks, hawks, and gulls, together with the crows, thrushes, and warblers. Of these only eleven fell in the rarest class, sixteen in the next, twenty-two in the next and nineteen in the most common. For variation in length and breadth together the average values from the totals shown in Table IV are -0 900, -2 750, -2 104 and +5 272. As eleven species are rather a small number for a class, the two least numerous classes may be thrown together with an average of -1 996. This differs from the value for the most abundant class by 7 268, or 16.7%. In the orders divisible into three abundance classes the average difference was 21.5%. It would seem at the least that differences in population size only apart from the many other factors at work, must account for difference of variability of the order of 20%.

As in the case of the division into three classes, the greatest contrast appears between the most abundant species and the next class, while among the rarer species differences in abundance are little associated with differences in variability. In our previous work with moths a similar phenomenon appears. The ten abundant species are more variable than the twelve 'common' species by 42%, while the twelve common species exceed thirteen species classed as less than common by little more than 14%. The phenomenon may, therefore, be a real one. If so, it indicates a very important conclusion, namely that rapid evolutionary progress is somewhat strictly confined to a small minority of very abundant species from which evolutionary radiation may later take place.

It is, however, at present possible that this inequality in the increase of variability as we pass from the less to the more abundant species is due to the defects of our knowledge. It is possible that the rarer species, though seldom taken to be very abundant, are more liable to be misclassified as to order of abundance among themselves. Such misclassification would greatly diminish the apparent increase in variability with increasing population. It is not impossible, likewise even if relative abundance were well determined among the rarer species, that their order of abundance is less



TABLE IV—COMPARISONS OF DEVIATIONS FROM GROUP MEANS OF ADJUSTED VARIABILITIES IN LENGTH AND BREADTH OF EGG FOR GROUPS DIVISIBLE IN FOUR ABUNDANCE CLASSES

Rarest	Variability in		Less rare	Variability in		Less common	Variability in		Commonest	Variability in	
	Length	Breadth		Length	Breadth		Length	Breadth		Length	Breadth
<b>Anser</b>											
Shelduck	-17.96	-15.17	Eider	9.07	-3.91	Wigeon	12.57	22.45	Mallard	-2.41	4.25
Graylag goose	17.75	24.03	Scooter	1.52	-6.41	Pochard	-2.01	-16.79	Teal	16.45	-0.49
			Goosander	-30.24	-15.90	Gadwall	-13.43	17.43	Pintail	5.36	28.37
			Red breasted merganser	-9.63	-13.82	Tufted duck	-8.80	-11.72	Shoveller	21.22	-12.31
<b>2 Ducks</b>	-0.21	8.86	<b>4 Ducks</b>	-29.28	-40.04	<b>4 Ducks</b>	-11.17	11.37	<b>4 Ducks</b>	40.62	19.82
<b>Accipitres</b>											
Golden eagle	7.08	17.56	Kite	4.78	-15.49	Merlin	-22.86	-14.11	Kestrel	-3.98	-12.27
Sea eagle	10.85	22.77	Honey buzzard	-11.41	-17.10	Peregrine	-11.61	-12.90	Sparrowhawk	27.41	27.42
						Hobby	-13.58	-3.98	Buzzard	-9.19	-0.49
						Hen harrier	22.53	8.61			
<b>2 Accipitres</b>	17.88	40.83	<b>2 Accipitres</b>	-6.63	-32.59	<b>4 Accipitres</b>	-25.52	-22.38	<b>3 Accipitres</b>	14.24	14.66
<b>Gravise</b>											
Great akua	-0.91	3.91	Great black backed gull	-21.84	-18.01	Black headed gull	-3.63	-5.13	Kittiwake	-2.98	-4.20
Arctic akua	-1.44	3.58	Little black backed gull	18.89	14.69	Common gull	7.61	19.83	Herring gull	4.68	23.16
Rosette tern	-9.86	-15.22	Sandwich tern	-6.95	-17.15	Arctic tern	-1.93	-0.67			
			Little tern	11.52	1.32	Common tern	6.84	-6.15			
<b>3 Gulls</b>	-12.21	-7.73	<b>4 Gulls</b>	1.62	-19.15	<b>4 Gulls</b>	8.89	7.88	<b>2 Gulls</b>	1.70	13.96



stable with the passage of time than is that of the really dominant species. For proportionate changes in numbers among the rarer species must be followed by far less important reactions on the ecological balance, than when dominant species are concerned. It would, for these reasons, be premature at present to assume that the association of genetic variability with abundance is so strongly confined to the more abundant species as would at first appear.

It has been essential, in order to demonstrate objectively the increase of variability with increasing population, that the variability should be unknown when the order of abundance within each group was assigned, and when the grouping was determined. One alteration has been made later, namely the separation of the grebes from the divers, which Mr. Alexander advised me should not be regarded as of the same natural order. This change diminishes the apparent effect, since the grebes are, on the whole, the more variable as well as the more numerous of the two groups. Once, however, the general effect has been demonstrated, it is permissible to consider what factors may have contributed to the more striking exceptions to the general rule. One such cause which should be noted as probably effective is change of population during the human period, for it is difficult to conceive theoretically that the variability should change much in response to change of population in a period so short as 10,000 generations. Any changes, therefore, in the relative frequencies of different species of the same groups, consequent upon the occupation and cultivation of land by men, may have produced comparatively large deviations from the general tendency.

Among rare species, showing relatively large variation, the most prominent is the graylag goose and the sea eagle. These seem, not improbably, birds which may have been relatively more abundant in the distant past. Without these two birds the least abundant class would be much the least variable. On the other side, almost the largest discrepancy among common birds is the house sparrow, which is less variable than the average of the finches, and must certainly have increased greatly in population with the increase in human buildings. The only other deviation so large among the common birds is the meadow pipit, but I do not know that this bird has been favoured by cultivation in comparison with the other pipits and wagtails.

With respect to classification, I have tried to restrict the comparisons in the tables of variability to birds which are really closely related. Of the picarian birds I have only used the woodpeckers and wryneck, since the kingfisher, swift, and nightjar seem to be insufficiently comparable with them, and the cuckoo must also certainly be excluded.

# 5—SUMMARY

Primary statistics and adjusted measures of variability are given for the length and breadth of eggs of 180 populations of British nesting birds

Comparisons within natural taxonomic groups show that the more abundant species are generally more variable than the less abundant species, in accordance with Darwin's generalization

The magnitude of the differences observable in the egg measurements of British birds is about 20 %

The excess variability *appears* to be strongly concentrated among the few most numerous species of each group, suggesting that evolutionary progress is much more rapid among these than among other species. This important indication may, however, be due to our comparative ignorance of the real relative numbers of the populations of the less numerous species

# 6—APPENDIX ON THE ANALYSIS OF COVARIANCE

The 161 species available for comparison within groups fall into twenty four groups, and leave therefore, 137 degrees of freedom for each of the four variates. The two independent variates  $L$  and  $B$  stand for the logarithms of the mean length and mean breadth for each species. The sums of squares of these within groups, together with their sum of products, supply the Information Matrix for the regressions, the elements of which are the coefficients of the equations of estimation by which the regressions are obtained

$$\begin{aligned} S(L - \bar{L})^2 &= 0.9322847, & S(L - \bar{L})(B - \bar{B}) &= 0.8546502, \\ S(L - \bar{L})(B - \bar{B}) &= 0.8546502, & S(B - \bar{B})^2 &= 0.7961181 \end{aligned}$$

Inverting this matrix we have the Multiplier Matrix

$$\begin{aligned} c_{11} &= 67.57225 & c_{12} &= 72.54029, \\ c_{12} &= 72.54029, & c_{22} &= 79.12968 \end{aligned}$$

If  $u$  and  $v$  stand for the two dependent variates, the logarithms of the variances of the lengths and breadths, the regressions of  $u$  on  $L$  and  $B$  will be given by

$$c_{11}S(L - \bar{L})(u - \bar{u}) + c_{12}S(B - \bar{B})(u - \bar{u})$$

and

$$c_{12}S(L - \bar{L})(u - \bar{u}) + c_{22}S(B - \bar{B})(u - \bar{u})$$

respectively, while those of  $v$  on the same pair of independent variates will be given by

$$c_{11}S(L - \bar{L})(v - \bar{v}) + c_{12}S(B - \bar{B})(v - \bar{v})$$

and

$$c_{12}S(L - \bar{L})(v - \bar{v}) + c_{22}S(B - \bar{B})(v - \bar{v})$$

The sums of products of  $u$  with  $L$  and  $B$  within groups are

2 4025188 and 2 1783253

giving the regressions

+ 4 327256 and - 1 909220

The sums of products of  $v$  with  $L$  and  $B$  within groups are

2 2034949 and 2 0493293

giving the regressions

- 0 236169 and + 2 320619

The sum of squares of  $u$  within groups accounted for by variation of  $L$  and  $B$  is found by multiplying the regressions by the corresponding sums of products and adding. The same method gives the sum of squares of  $v$  and the sum of products of  $u$  and  $v$  we have therefore the following analysis of variance

	Degrees of freedom	$u^2$	$uv$	$v^2$
Regression	2	6 237411	4 487663	4 235315
Remainder	135	2 642530	2 274683	3 739038
Total within groups	137	8 879940	6 762345	7 974354

The mean squares and mean product from this table are

	$u$	$v$
$u$	0 0195743	0 0168495
$v$	0 0168495	0 0276966

which it is convenient to refer to as the matrix of residual covariance

It appears that whereas before correction the variance in length is more variable than that in breadth after correction the reverse is true. More over after full allowance has been made for the mean length and breadth the variability of length and breadth is still highly correlated showing that species more variable in egg length are also generally more variable in egg breadth. For 100 eggs measured sampling error will give to the logarithms (to the base 10) of the variances in length and breadth the sampling variance

$$\frac{2}{100(\log 10)^2} = 0.003772$$

while the contribution to the sampling covariance will be smaller by reason of a factor  $\rho^2$  the square of the correlation coefficient between the lengths and breadths of individual eggs a factor which seldom exceeds 0.2. Even in the case of  $u$  therefore the sampling error accounts for only about one fifth of the variance observed this is however a sufficiently large proportion

to show that any much smaller number of eggs might have been insufficient to display the real differences in variability

The standard errors of the regression coefficients and of any linear compounds of them are derived from the multiplier matrix and the matrix of residual covariance. Thus the sampling variance of the regression of  $u$  on  $L$  4.327 is

$$0.0195743c_{11} = 1.3227$$

giving a standard error 1.150 so that the regression is on the verge of being significantly greater than 2

For the sum of the regressions of  $u$  on  $L$  and  $B$  2.418 we use instead of  $c_{11}$  the factor  $c_{11} + 2c_{12} + c_{22} = 1.62135$  and obtain the standard error 0.1781. The significance of the small excess 0.418 is thus higher than that of the large excess 2.327 in the single regression. Equally for change of shape without change of volume we need the difference between twice the regression on  $L$  and the regression on  $B$  for which the appropriate factor is  $4c_{11} - 4c_{12} + c_{22}$  or 639.57984. We may thus combine the independent variates in any way we please.

Combination of the dependent variates is carried out by means of the residual covariance matrix. Thus to test the significance of the sum of the regressions of  $u$  and  $v$  we should use the residual variance of  $u + v$

$$0.0195743 + 2(0.0168495) + 0.0276966 = 0.0809699$$

while for the difference we have the higher precision represented by the residual variance of  $u - v$

$$0.0195743 - 2(0.0168495) + 0.0276966 = 0.0135719$$

Thus whereas the sum of the regressions of  $u$  is  $2.418 \pm 0.1781$  so that the excess over the value 2.0 is significant the regressions of  $v$  amount only to 2.084 with about the same precision which is certainly not a significant excess. Adding the two together we have  $4.502 \pm 0.3623$  where the standard error is derived from the sampling variance

$$0.0809699 \times 1.62135$$

The regressions of  $v$  though together exceeding 2.0 do not do so sufficiently to increase the significance of the discrepancy.

With regard to change of shape without change of volume we shall need twice the regression on  $L$  less the regression on  $B$ . For  $u$  this is  $10.564 \pm 3.538$  where the sampling variance is

$$0.0195743 \times 639.57984$$

so that the value for  $u$  does not significantly exceed 4. For  $v$  similarly we

have  $-2.793 \pm 4.209$  showing a quite insignificant difference from the value  $-2$ . Using the difference  $u-v$  we have consequently  $13.357 \pm 2.946$ , since the sampling variance is

$$0.0135719 \times 639.57984$$

The excess over 6 being 7.357 is thus considerably greater than twice the standard error and is clearly significant.

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581.035.1

## The Transmission of Light and Total Radiation by Leaves

By W. R. G. ATKINS, SC.D., F.R.S.

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In the course of work upon the colour composition of light in woods (Atkins and Poole 1931; Atkins, Poole and Stanbury 1937) it became evident that when the daylight factor (viz. the percentage of the external diffuse illumination) was low there was always a marked difference between the spectral composition of the light in the wood and that of its source. This is obviously due to the fact that the proportion of the light transmitted by the leaves or reflected from leaves and branches is greater when the daylight factor is low. It became of interest therefore to measure the transmission of leaves.

Such determinations might be carried out photographically with a spectrum projector or similarly using a thermopile to record the transmission. Alternatively a spectrophotometer might be used. It seemed simplest however to make the determinations photoelectrically in the open using the cells, light filters and thermopile already used for measuring the light and energy in and around the woods.

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For measuring the currents portable microammeters of low resistance gave adequate sensitivity but with heavily absorbing light filters an instrument with the Onwood type of suspension and having 312  $\Omega$  resistance was used

For determinations throughout the visible spectrum the selenium rectifier cell served well the readings being corrected for the curvature of its illumination current relation Its sensitivity is low however in the extreme red and it has but little infra red sensitivity The red sensitive type X cell of the Oxford Instrument Co was accordingly used for this region and the Moll solarimeter was used for total energy below the region cut off by glass

The light filters were of Jena glass 2 mm thick but for green a 4 mm Corning glass was used in order to reduce the red transmission to a negligible amount

Transmissions were always measured for diffuse light namely with the leaf under the opal diffusing surface This was done so that the angular distribution of the incident light should always be the same since it is variable in the open When the Moll solarimeter was used the leaves had to be spread over the glass hemispherical cover so sunlight would be incident more or less normally

It was desirable to use large leaves so as to cover the cell apertures and the solarimeter both on account of the size of their leaves and because these have so often been used in physiological work *Syringa vulgaris* was selected as a tree and *Tropaeolum major* as a herb Leaves of the latter were about 0.13 mm thick when from a light green variety and about 0.18 mm thick when from a much darker green variety The *Syringa* leaves were 0.12–0.16 mm thick The leaves were placed with their upper surface upwards but for *Tropaeolum* at least the position was proved to make but little difference with leaves having a markedly glossy upper surface the difference would probably be considerable

The table shows how very different is the absorption in various spectral regions In general the *Syringa* leaf transmits about the same amount as does the darker *Tropaeolum* leaf but the lighter leaf transmits about twice as much in the short wave region

In spite of the fact that a leaf looks green when held up against the light we see that it transmits only 4–10 % of the green Taking the entire red it transmits 7–13 % but the relatively greater sensitivity of the eye for green causes us to see the leaf as green the blue transmission being negligible The deep red however is relatively freely transmitted and the total energy transmission is somewhat similar since the red and the infra



red constitute such a large proportion of the total. It may accordingly be seen that the richness of forest light in red is due to its high transmission by the leaves, since chlorophyll transmits freely in this region. The figures obtained for total energy transmission, 38% in each case, are close to the value 35%, found by Brown and Escombe (1905) for the leaf of *Polygonum Weyrichii*. The determinations in the other spectral regions are in general

TABLE

6-9 August 1935. Percentage transmission of leaves as found using selenium cell, S, or type X cell. Conditions: clear blue sky, cloudless, no direct sunlight. Using a Moll solarimeter in bright sunlight and skylight the lighter green *Tropaeolum* leaf transmitted 38.5% and the *Syringa* leaf 38.2% of the energy from the whole spectrum, which was about 70 mW/sq. cm.

Instrument and filter	Range in mμ	<i>Tropaeolum</i>		<i>Syringa</i>
		Darker	Lighter	
S, opal always	320-800*	3.9	9.6	4.7
S, BG 12	350-500†	0.1	0.5	0.2
S, Corning green	460-600‡	3.8	10.4	5.0
S, GG 3	410+	4.7	9.8	4.9
S, GG 11	500+	6.3	13.3	6.7
S, OG 2	550+	7.0	15.9	7.1
S, RG 1	600+	6.9	12.7	6.9
S, RG 2	630+	7.2	13.5	6.8
S, RG 5	675+	14.3	18.0	13.7
S, RG 8	700+	38.8	44.0	34.8
X, RG 7	850+	—	56.0§	—

\* Maximum sensitivity at 595 mμ

† Maximum transmission at 430 mμ

‡ Maximum transmission at 530 mμ

§ Transmitted 67.8% in bright sunlight and skylight

agreement with the more extensive measurements of Seybold (1932), effected by means of a Linke thermopile with which I have since become acquainted. A more detailed study by the photoelectric method would be possible were a second cell to be used to measure the variation in the sky illumination from minute to minute, so that all readings with filters could be reduced to a definite intensity, with this knowledge the transmission of fairly narrow spectral bands could be determined by means of the GG, OG and RG series of filters, which have sharp spectral limits.

I am indebted to the Government Grant Committee of the Royal Society for defraying the cost of all the instruments used.

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### SUMMARY

Leaves of *Tropaeolum majus* and *Syringa vulgaris* were found to transmit 38% of the energy from mixed sunlight and skylight and *Tropaeolum* transmitted 56–68% of the near infra red

Transmission in the visible region beyond  $700\text{m}\mu$  was found to be as high as 35–44% though only 4–10% in the green and less than 0.5% in the blue. These figures show why the spectral composition of the light in woods is so rich in red when determined at sites having a low daylight factor

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532 62

## Molecular Interaction in Monolayers I—Complexes between large Molecules

BY J H SCHULMAN AND E K RIDEAL F R S

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(Received 14 October 1936)

In previous communications (Rideal *et al.* 1933, 1935) from this laboratory it has been shown that the course of chemical reactions proceeding in monomolecular films or monolayers at air liquid interfaces can be followed by means of observation of the changes in phase boundary potential and surface pressure or tension. It seemed possible that a further advance in the elucidation of the complex mechanism of lytic sensitizing agglutinating and immunity reactions might be made by a closer study of the behaviour and interaction of a number of biologically active substances in the form of monolayers especially as it appears that several of these biological processes take place under conditions similar to those existing in the films

It is found that if an extremely dilute solution of a suitable molecular species be injected under a monolayer of another under the correct interacting conditions a phenomenon which we may term film penetration is observed. The chief characteristics of film penetration are an immediate large rise in surface pressure of the film, a decrease or increase of the surface potential according to the magnitude of the dipole moments of the penetrating polar group compared to that of the film forming molecule, and the strong stability of the resultant new film. It appears that the process of penetration is completed when a mixed film consisting of equimolecular proportions of the original film material and the penetrating substance is formed. The final value of the phase boundary potential is found to correspond exactly with that shown by the equimolecular mixed film spread on the surface and compressed to maximum pressures.

Closer investigation reveals the fact that the process of film penetration and the formation of the equimolecular mixed film is due to the mutual interaction of the two molecular species resulting in the formation of complexes. Some strongly interacting molecules form complexes which are sufficiently stable to persist in bulk phase, and mixed films composed of the same molecules made by mixture or by penetration are characterized by a remarkable stability much greater than that possessed by films of either of the constituents alone. Since many natural membranes probably consist of mixed monolayers of orientated molecules, this observation might throw some light on their mechanical stability.

It was found that where strong complex formation between two substances could be identified by the film technique of penetration, this was reflected in their behaviour in bulk to form new compounds. Likewise where complex formation was known to exist in bulk, these systems were found to exhibit excellent film interactions.

On the whole a remarkable specificity between complexes is shown by these methods. A grading of the extent of interaction between molecules is readily achieved by observation of the film penetration and the stability of the mixed films, but estimation of the extent of interaction for bulk complexes is much more difficult. The inhibition or sometimes acceleration of haemolysis by lytic substances after complex formation can be used for the extent of interaction, whilst prevention of the formation of precipitates of insoluble heavy metal salts and the dispersion of the components to form soluble complexes provide other methods.

The factors governing film penetration are found to be identical with those governing complex formation in mixed monolayers, and it is possible to analyse the mechanism of film penetration in detail.

In the first place the original monolayers may be regarded as being constituted of molecules possessing a hydrophobic portion or "tail" and a hydrophilic "head" or polar group immersed in the substrate. The substance injected underneath the film may likewise be regarded as made up of a hydrophobic tail and a polar hydrophilic head.

The first condition for penetration is that there shall be some mutual interaction between the polar heads of the two molecular species, thus organic acids injected under a film of a long chain acid will not affect the phase boundary potential or the properties of the film, but the injection of organic acids is accompanied by marked changes in the characteristics of a film of cetyl alcohol when injected under the latter. The results of a number of experiments of this type lead to the conclusion that the primary act in the process of penetration is the anchoring of the injected molecules by a suitable polar group to a polar group of the film forming material which is immersed in the water. The extent of this interaction between the head groups forming the complexes in the mixed film depends on the nature of the interacting polar groups.

A number of substances, which we may conclude from the change in phase boundary potential on injection do actually anchor themselves in this way, are found not to exhibit any subsequent penetration of the film and do not increase the surface pressure of the film, thus gallic acid and tannic acid are adsorbed beneath monolayers of proteins but do not penetrate them. These reactions present several points of interest which we shall have occasion to refer to later.

It is found that the second condition for penetration is that there shall be a hydrophobic tail to the penetrating molecule which adheres or interacts by van der Waals forces with the hydrophobic tail of the film forming material. Thus, whilst short chain acids (e.g. pelargonic acid) penetrate a film of cetyl alcohol but slowly, myristic acid penetrates with great rapidity. Variations in the nature of either the polar groups or of the hydrophobic portions (e.g. by the insertion of a double bond) of either the film forming or film penetrating material or small alterations caused by change in environment (e.g. pH or salt concentrations) frequently exert such a remarkable influence both on the stability of the mixed film and on the ease and extent of penetration as to give the impression of the complex possessing almost specific properties. This sensitivity goes hand in hand with the breakdown of the complexes which these substances form in solution.

A "complex" is an association product between at least two substances, having chemical characteristics different from those of either of the com-

ponents alone, but which usually separates into its original components on crystallization from solution

Some complexes (e.g. digitonin cholesterol) crystallize from solution without dissociation but are very labile, for instance, the digitonin-cholesterol complex crystallizes as such from alcohol but will dissociate if heated in solution (Windaus 1909)

The vertical component of the apparent molecular electric dipole moment is  $\mu$  as given in the Helmholtz equation

$$\Delta V = 4\pi n\mu$$

$\mu$  is the summation of a number of factors due to the insertion of a dipole into the surface of an aqueous solution. This has already been described by Schulman and Rideal (1931) and Schulman and Hughes (1932)

#### EXPERIMENTAL

The materials to be examined in the form of monolayers were dispersed on the surface of suitable substrates in a Langmuir trough, the phase-boundary potentials measured by the method described by Schulman and Rideal. Since changes in surface pressure of the films were to be investigated, two rings attached to two chainomatic balances were placed on either side of a central barrier across the Langmuir trough. A solution of the reacting substance of the order of  $10^{-4}\%$  is injected and mixed into the underlying solution and its action on the two films on either side of the barrier takes place under identical conditions, and thus a strict comparison can be obtained. The surface pressures were measured by the method of Cary and Rideal (1925) in that the ring was brought to a certain height just above the liquid without breaking the surface, any difference in height being optically magnified with the help of a small mirror attached to the arm of the chainomatic balance.

Should the ring break the surface, some few minutes must elapse before conditions return to normal. In some cases changes of surface pressure of some 60 dynes/cm. take place in less than 2 min. and it was found advisable to make a preliminary adjustment of the chainomatic balance before the reaction commenced. The dimensions of the chains and rings were so chosen that 1 cm. of movement of the chain was equivalent to 1 dyne/cm. change in surface tension. Both platinum and copper rings were employed and calibration was carried out with films of known surface pressure with water and by weighing.

Apart from the films of oleyl alcohol, lecithin, and the proteins, the substances described in this communication were found to form condensed

films The state of compression was found to make but little difference to the penetration, except when the latter was weak Under these circumstances it was found possible to prevent penetration by suitable increase in the surface pressure A pressure of 10–12 dynes/cm was usually chosen if the film could be compressed to that value without collapse This value of 12 dynes was approximately the surface tension lowering of the free surface of the aqueous solution, due to the injection of the cetyl sulphate or saponin into the underlying solution at the described concentrations (figs 1 and 2) The substrate consisted generally of M/25 potassium hydrogen phosphate buffer of pH 7.2, made up according to the directions of Clark and Lubs

It is interesting to note that the phenomenon of penetration is not materially affected by the initial or final physical state of the monolayer, thus a film of ergosterol originally a solid becomes liquid on injection of either saponin or sodium cetyl sulphate into the substrate, whilst a liquid film of cholesterol becomes solid on injection of saponin or digitonin These changes in characteristics are exemplified in Table I

TABLE I

Substance	Substrate	State	Substrate	State
Sphingomyelin	Saponin	Liquid (no change)	Sodium cetyl sulphate	Liquid solid
Cholesterol	„	Liquid solid	„	Liquid (no change)
Ergosterol		Solid liquid	„	Solid liquid
Caleiferol		Liquid (no change)	„	Liquid (no change)
Oleyl alcohol	„	Liquid (no change)	„	Liquid (no change)
Cholesterol acetate	„	Liquid (no change)		Solid liquid
Cetyl alcohol	„	Liquid (no change)	„	Liquid solid
Cetyl phenol	„	Liquid (no change)	„	Solid liquid
Lecithin	„	Liquid (no change)	„	Liquid (no change)

It has been shown in a previous communication (Schulman and Hughes 1935) that the characteristics of mixed films are dependent on the extent of dipole and van der Waals interaction, the association of two molecules being graded in their ability to form stable mixed films If the association be weak, one component is squeezed out of the surface on compression of the mixed film, with a strong interaction the mixed film possesses charac-

teristics entirely different to either of the two components alone. With weak association the mixed films show no dipole interaction, the resultant surface potential being the mean of the two dipoles according to their number. Whereas, when association is shown, the resultant surface potential reveals a mutual interaction of the dipoles of the film forming molecules.

As an example of weak association the behaviour of oleic acid and cetyl alcohol at pH 2 is given. On compression of an equimolecular mixed film oleic acid is compressed out of the surface and the residual film consists ultimately of the pure alcohol. No dipole interaction is observed.

As an example of stronger association the ionized acid at pH 7.4 may be cited. Here the vertical component of the moment of the  $-\text{COO}^-$  ion is much smaller (i.e. 0.1 Debyes) than that of the alcohol (0.21 Debyes) and interaction in the mixed film is observed both by the formation of a new stable mixed film and by the surface potential which reveals deformation of the dipoles. Here the assumption has been made that the dipole moment of one of the components has not altered although the molecule is now at areas which on its own as a film it cannot exist. This assumption is supported by the fact that where no association takes place the dipole moment remains the same although the molecule is now at areas to which it cannot be compressed or expanded on its own.

pH 7.4	Surface potential	
	Obs	Calc
At area $25 \text{ \AA}^2$	200	230
$20 \text{ \AA}^2$	230	292

Another example of weak association is triolein and cetyl alcohol. At the highest compression where the two components can exist together before the alcohol ejects the ester from the surface no distortion of the dipoles is noticed.

At 1/3 ratio pH 2.0	Surface potential	
	Obs	Calc
At $40 \text{ \AA}^2$	383	382

With tripalmitin and oleic acid some weak association is observed at pH 7.4

At 1/3 ratio	Surface potential	
	Obs	Calc
At $32.2 \text{ \AA}^2$	340	380
$50 \text{ \AA}^2$	230	245
At 1/1 ratio		
At $36.2 \text{ \AA}^2$	460	572

With sphingosine and cetyl alcohol where strong interaction is noticed the following distortion of the dipoles is noticed

pH 7.4	Obs	Calc
25 Å <sup>2</sup>	400	422
20 Å <sup>2</sup>	470	518
pH 2.0		
20 Å <sup>2</sup>	495	538
28.6 Å <sup>2</sup>	400	376

We note that the extent of compression of the films influences the extent of dipole interaction

These figures were taken from the experiments described by Schulman and Hughes (1935 pp 1245-7)

To show that upon injection of one substance underneath another which results in penetration showing strong association of polar groups the resultant surface potential is that of the equimolecular mixture of the two components the following values are given

The observed surface potential of an equimolecular mixed film of sphingosine and hexadecyl alcohol at maximum compression at 20 Å<sup>2</sup> was 475 mV. Injection of sphingosine under cetyl alcohol film of 422 mV (at pH 7.2) resulted in a rise of the surface potential to 475 mV. Upon injection of sodium oleate under a compressed film of cetyl alcohol at pH 2.0 no change in the surface potential is observed. Interaction is weak and the dipoles of the two substances are identical but at pH 7.4 the oleate ion of much smaller moment penetrates the film to lower the surface potential to 270 mV. The value of the surface potential of the equimolecular mixed film of the two components at pH 7.4 at maximum compression is 270 mV.

We have examined a number of such cases less quantitative in nature showing very similar results. The substances in these cases were too soluble to exist as films on their own but only form Gibbs layers thus their electric moments are difficult to determine. Psychosine which possesses a high electric moment and which is very reactive in penetration always raises the surface potential of most film forming substances and the relatively soluble sodium cetyl sulphate possessing a comparatively small moment lowers the surface potential of most films on penetration. It is interesting to note in this case that sodium cetyl sulphate lowers the surface potential of a fatty acid on acid substrates but on neutral substrates where the ionized carboxyl has a very small moment e.g. lower than the sulphate it raises the potential of the fatty acid film.



Psychosine sulphate injected into the underlying solution at a concentration of  $ca\ 10^{-4}\%$ , will raise the surface potential of the following substances within a few minutes at  $pH\ 7.4$  of

Lecithin	from 360 to 419 mV,
Tripalmitin	„ 650 „ 703 mV,
Cetyl alcohol	„ 400 „ 536 mV,
Cholesterol	„ 390 „ 518 mV,
Palmitic acid	„ 396 „ 644 mV ( $pH\ 2.0$ )

Sodium cetyl sulphate injected into the underlying solution at a concentration of  $ca\ 10^{-4}\%$  will lower the surface potential at  $pH\ 2.0$  of palmitic acid from 390 to 264 mV at  $pH\ 6.5$  will raise the surface potential of palmitic acid from 280 to 324 mV Sodium cetyl sulphate injected under similar conditions will lower the surface potential of palmitic acid at  $pH\ 2.0$  from 390 to 280 mV and will raise the surface potential at  $pH\ 6.6$  from 260 to 346 mV

To extend our knowledge of this mechanism of penetration and complex formation to substances of more immediate biological interest, two series of experiments were carried out one to determine the degree of polar association and secondly to trace the influence of an alteration in the hydrophobic character of the non polar portion of the molecule on the ease of penetration and stability of the resulting complexes

In fig 1 are shown the effects of injecting 5 mg saponin (or digitonin) into 300 c.c of  $M/25$  phosphate buffer at  $pH\ 7.2$  under condensed films of various substances placed on the surface and each compressed to a surface pressure of 10 dynes/cm The resulting lowering of the surface tension by the saponin solution alone was found after a period of several hours to be 15 dynes/cm The remarkable effects of injection of the saponin and digitonin on a film of cholesterol are noted in the curve There is an immediate rise in surface pressure to some 60 dynes/cm the liquid cholesterol film becomes solid and there is an accompanying fall in phase boundary potential of 100 mV from 400 to 300 If the alcoholic group of the cholesterol be replaced by the acetate which contains an ester polar group possessing a weaker association for the acid group in the saponin, the rise in surface pressure is strongly inhibited and rises only some 7–10 dynes/cm after 15 min Again if the hydrophobic portion of the cholesterol molecule be altered as in calciferol, only a relatively small penetration by the saponin is obtained (a change of 10 dynes/cm)

Digitonin differs from saponin in its activity in that it is more effective in its action on these films, although with the same ratio of surface pressure

differences. It will penetrate films of long-chain alcohols which saponin does only to a small extent. The latter exhibits (fig. 1) a slight penetration into films of alcohols, phenols, sphingomyelin and lecithin, and although the polar interaction is small it is detectable in the alcohols by causing a

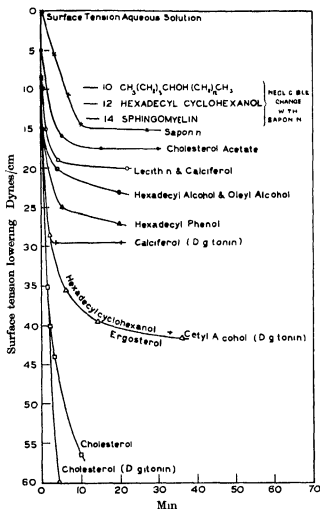


Fig. 1—Saponin  $1.6 \times 10^{-3}\%$  injected at pH 7.2 under various films

change of 30 mV in the surface potential. This weak penetration by saponin can be completely inhibited by prior compressing of the films of the alcohols, phenols and lecithin to the critical pressures which are obtained after injection of the saponin under the films at the 10 dynes/cm pressure. This association is even weaker than that of the oleic acid and

esters where one component is squeezed out of the surface on compression of the mixed film (Schulman and Hughes 1935)

Thus if films of calciferol lecithin cetyl alcohol and oleyl alcohol be compressed to 22 dynes surface pressure and saponin be now injected into the underlying solution there is no change in the surface pressure of films of these substances. If on the other hand films of cholesterol be compressed to their maximum compression before collapsing *ca* 30 dynes no inhibition of the penetration by the saponin is effected and the pressure immediately rises to that of the mixed film which being more stable stands pressure of *ca* 60 dynes (as measured by the ring method)

This marked penetration of films of cholesterol and ergosterol (and no other films) by saponin as well as the vigorous penetration of hexadecyl cyclohexanol and cetyl alcohol by digitonin is reflected in the well known association of these substances to form complexes in bulk solution

To show that complexes could be formed in a similar manner by a simple suitable choice of a polar group attached to an appropriate hydrophobic group a dilute solution of sodium cetyl sulphate was injected (fig 2) under films of these same substances as were reacted upon by saponin. 1 mg was added to the 300 c.c. of underlying solution of pH 7.2

The acid sulphate group is more reactive than the polar group in saponin or even digitonin. The soap itself only lowers the surface tension at this concentration about 12 dynes/cm. in about 20 min. whereas the surface pressures of 10 dynes/cm. of films of cholesterol ergosterol and cetyl alcohol are all increased some 45 dynes/cm. in a few minutes. As with the saponins the weakening of the alcoholic polar group by ester formation or insertion of a double bond into the hydrophobic portion of the cholesterol molecule strongly inhibits the ease of penetration of the films. Thus cholesterol acetate and calciferol are much less affected by injections of sodium cetyl sulphate into the underlying solutions changes of 20 dynes surface pressure being observed after 30 min. for these two substances.

The effect of a change in the hydrophobic portion of the molecule is shown clearly in the weakness of the penetration of a film of oleyl alcohol as compared to one of cetyl alcohol (fig 2). Thus the 10 dynes surface pressure of a film of cetyl alcohol is changed to 58 dynes within a few minutes when 1 mg. of sodium cetyl sulphate is injected into 300 c.c. of underlying solution. This compares with a change of only 23 dynes surface pressure for the oleyl alcohol film in half an hour. As with calciferol the simple insertion of a double bond greatly weakens the association of the hydrophobic groups and in fact is equal in effect to the changing of the alcoholic polar group to an ester group. This would explain the inability

of the saponin to form complexes in bulk with calciferol or penetrate its films. Here the inhibition of penetration by prior compression of the films, where weak penetration is observed, is again noticed as with saponin

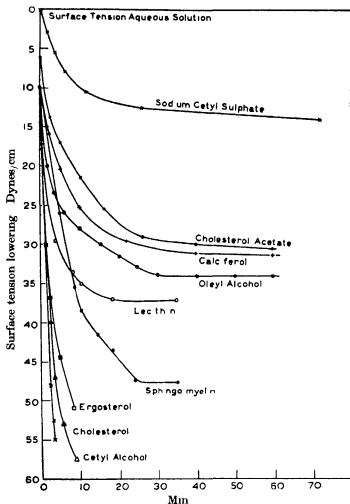


Fig. 2—Sodium cetyl sulphate  $3.3 \times 10^{-4}\%$  injected at pH 7.2 under various films, at 10 dynes surface pressure

Thus, if films of calciferol or oleyl alcohol be compressed to 30 dynes pressure, no further change in surface pressure is observed on injection of the sodium cetyl sulphate into the underlying solution. It is interesting in this connexion to observe that maximum compression of a film of cetyl alcohol does not stop the penetration of cetyl sulphate, whereas a compression of

22 dynes completely stopped the penetration of this film by saponin. The strong penetration of sphingomyelin films (fig 1) which contain the very

OH

reactive sphingosine polar group ( $-\text{CH}=\text{CH}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{NH}_2$ ) was to be expected and might be biologically significant whereas the lecithin film containing an unsaturated long chain ester is far less active

#### HAEMOLYSIS EXPERIMENTS

To demonstrate and grade the reactivity of these groups in solution the following experiments were carried out

Equimolecular mixtures of the reacting substances (one being sodium cetyl sulphate) were made up and dissolved in alcohol. They were then diluted with saline until the mixture contained 1 mg/c.c. of the sodium cetyl sulphate (Tables II and III)

Haemolytic experiments were carried out with the mixtures on 10 or 5% red cell suspensions. As with saponin and cholesterol the haemolytic activity of sodium cetyl sulphate is very nearly completely inhibited by cholesterol, only slightly by calciferol and not at all by cholesterol acetate. The haemolytic activity is not inhibited by the addition of hexadecyl cyclohexanol and long chain alcohols, although as we have seen, strong penetration of alcohol films takes place.

That definite complexes are formed between the cetyl sulphate and the alcohols can be shown by the fact that a completely insoluble substance such as hexadecyl cyclohexanol with a very sparingly soluble substance such as sodium cetyl sulphate form completely clear solutions when they are mixed in equimolecular proportions.

The fact that they are lytic is probably due to the stronger association of the cetyl sulphate to the cholesterol or protein in the membrane of the red cell than to the long chain alcohol.

On weakening the polar association of the acid group in the long chain to the OH group in the cholesterol by replacement of the sulphate by a carboxyl as in palmitic acid, no inhibition of the haemolysis by sodium palmitate takes place. This, as we shall see, is probably due to the stronger association of the carboxyl fatty acid to insoluble proteins on the membrane of the red cell and not to the cholesterol content of the cell. In a previous communication it has been shown (Schulman and Hughes 1935) that the fatty acid content of a protein fatty acid mixture in solution is completely removed by denaturing a portion of the protein and separating it from the soluble protein.

TABLE II

	Time	1/1	1/2	1/4	1/8	1/16	1/32
Sodium cetyl sulphate + cholesterol 1 mg. soap + 2 mg cholesterol in 1st tube at 37° C + 1 c c 10% sheep R C	1½ hr	-	-	-	-	-	-
	3½ hr	-	-	-	-	-	-
	O N *	+ ±	-	-	-	-	-
Sodium cetyl sulphate 1 mg in 1 c c saline in 1st tube + 2 c c saline at 37° C	1½ hr	C	+ + + ±	+ +	+	±	-
	3½ hr	C	C	+ + + ±	+	±	-
	O N	C	C	C	+	±	-
Sodium cetyl sulphate + cetyl alcohol. Equimolecular mixture (cetyl alcohol alone, no effect) Temperature 37° C	1½ hr	C	C	C	+	-	-
Dodecyl alcohol 1 mg in 1st tube	1 hr	C	+ + +	+	±	-	-
	2 hr	C	C	+ + ±	+	-	-
	3 min	-	+	-	-	-	-
	10 min	- ?	C	+ +	+	-	-
	1 hr 37° C	C	C	+ +	+	-	-
	3 hr 37° C	C	C	C	+ + + ±	-	-
Ethyl n-undecyl carbinol + sodium cetyl sulphate equimolecular mixture dil to 1 mg soap in 1st tube	O N *	C	C	C	+ + + ±	-	-
	3 min	C	C	+ +	+	+	+
	10 min	C	C	+ +	+	+	+
	1 hr 37° C	C	C	C	+ +	+	+
	3 hr 37° C	C	C	C	C	+ + +	+
	O N *	C	C	C	C	C	+
Ethyl n-undecyl carbinol 1 mg in 1st tube	3 hr 37° C	±	-	-	-	-	-
	5 hr 37° C	+ + +	+	-	-	-	-

\* O N = over night at room temperature

C = Complete haemolysis

TABLE III

	Time	1 1	1 2	1 4	1 8	1 16	1/32
Sodium cetyl sulphate + cholesterol 1st tube contained 1 mg cetyl sulphate 2 mg cholesterol 2 c c saline 1 c c 10% R C	65 min	-	-	-	-	-	-
Sensitized sheep R C Temperature 37° C	2 hr	+++	+	-	-	-	-
Sensitized sheep R C Temperature 37° C	O \ *	+++	+	-	-	-	-
Sodium cetyl sulphate + sensitized sheep R C Temperature 37° C	30 min	C	C	++	-	-	-
Sodium cetyl sulphate + sensitized sheep R C Temperature 37° C	1 1/4 hr	C	C	C	++	+	+
Sodium cetyl sulphate + cholesterol normal sheep R C	1 hr	++	-	-	-	-	-
Sodium cetyl sulphate + cholesterol normal sheep R C	O \ *	+++	+	-	-	-	-
Sodium cetyl sulphate + normal sheep R C	30 min	++	+	-	-	-	-
Sodium cetyl sulphate + normal sheep R C	1 1/4 hr	++	+	C	++	+	-
Sodium cetyl sulphate 1 mg in 1st tube + 2 c c saline 1 c c 5% rabbit R C	2 min	C	C	C	++	+	-
Sodium cetyl sulphate + cholesterol 1 mg soap in 1st tube at 18° C	10 min	C	C	C	C	++	-
Sodium cetyl sulphate + cholesterol 1 mg soap in 1st tube at 18° C	2 min	-	-	-	-	-	-
Sodium cetyl sulphate + cholesterol acetate Temperature 18° C	50 min	+++	+	-	-	-	-
Sodium cetyl sulphate + cholesterol acetate Temperature 18° C	2 min	C	C	C	++	-	-
Sodium cetyl sulphate + cholesterol acetate Temperature 18° C	10 min	C	C	C	C	-	-
Sodium cetyl sulphate + calciferol Temperature 18° C	2 min	C	C	++	-	-	-
Sodium cetyl sulphate + calciferol Temperature 18° C	30 min	C	C	C	+	-	-

\* O N = over night at room temperature

C = Complete haemolysis

All equimolecular mixtures

We thus see that one of the partners in these complexes may be regarded in the light of a carrier for the other substance forming the complex and the structure can be broken as soon as one of the polar groups (that of the substance carried) comes in contact with a stronger associating polar group, such as cholesterol in the red cell membrane. In fact such substances which are poorly lytic owing to their insolubility, as are the long chain alcohols, become very lytic when associated with a stabilizing group such as the sulphate. The lytic properties of an equimolecular mixture of a long chain alcohol and sodium cetyl sulphate are nearly double that of the sulphate alone the alcohol being too insoluble on its own to be active.

The action of the alcohol is almost certainly on the protein content of the red cell membrane, since films of egg albumen and of gliadin are readily penetrated by tetradecyl alcohol when injected into the underlying solution.

In Tables II and III are shown the inhibiting effects due to the association of polar and non polar groups with one another in lytic substances on the lysis of red cells.

We may conclude that the existence of these complexes, formed by the mutual association both of polar groups and of hydrophobic groups in two different molecular species may be demonstrated by the following physical chemical criteria: penetration of monolayers; association in mixed films; inhibition or sometimes increase of lytic activity; mutual dispersion in aqueous solution and in certain cases complexes may be formed by crystallization of the two ingredients from their mixed alcoholic solutions. Complex formation can inhibit the formation of insoluble precipitates, thus in an aqueous solution of an equimolecular mixture of cetyl sulphate and cholesterol the precipitation of the cetyl sulphate by silver nitrate does not take place, although sodium cetyl sulphate and cholesterol crystallize out separately from their alcoholic solution. The digitonin and cholesterol complexes remain stable on crystallization.

We note that the  $\text{SO}_3\text{Na}$  polar group exhibits a strong association with the sterol:  $\text{OH} > \text{long chain OH} > \text{phenol OH} > \text{long chain ester} > \text{sterol ester}$ , whilst the association of the acid radical is weakened by changing to the carboxyl ion such that it now only forms dispersing complexes when attached to the same hydrophobic group, viz  $\text{CH}_3(\text{CH}_2)_{15}$ . The unionized carboxyl group forms complexes which are still less stable.

In respect to the association of the hydrophobic portions of the molecules we note that

(1) The condensed ring\* systems and saturated long chains exhibit a *strong* (long chain sulphate + cholesterol) interaction

Cyclopentano hydrophenanthrene



(2) The unsaturated condensed ring system and saturated long chains show a *weak* interaction there is only a weak penetration of calciferol by a long chain sulphate in spite of the strong polar interaction

(3) A saturated long chain interacts strongly with a saturated long chain but not so strongly as (1) even when a strong polar association is provided

(4) A saturated long chain exhibits a weak interaction with an unsaturated long chain when the double bond is in the middle of the long chain

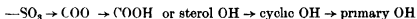
The reactive polar group in digitonin is probably the unsaturated carboxyl attached to the condensed ring hydrophobic group It exhibits a strong association with sterol OH > long chain hydrocarbon hexanol > long chain OH and a negligible one with the sterol ester

The polar group in saponin exhibits a strong association with a sterol OH a weak one with a long chain phenol or alcohol > sterol ester > lecithin > long chain hexanol

In the latter case the reactions are partly masked by the varying polarity of the cholic group in saponin and digitonin saponin being more polar and not combining to form complexes with any substance mentioned except cholesterol

We may conclude that to form stable complexes at least two strong points of contact must be established between two molecules strong polar but weak hydrophobic association weakens any form of complexes or dispersing properties but a weak polar association and a strong hydrophobic adhesion can produce weak complexes in the form of dispersion complexes

The chief characteristic of molecular association is their sensitivity to such small changes in polar reactivity as in the change of acidity from



or the change in polarity produced by the insertion of a double bond Again we note the great stability of mixed films which will stand compression up to 60 dynes/cm these do not collapse but can be pushed over the edges of the surrounding paraffin glass barriers whereas the components by themselves very rarely stand pressures greater than 35 dynes/cm

Finally a penetrating molecule which has a strong adhesion to the film forming molecule will penetrate instantly a solid condensed film against a pressure of 35 dynes/cm to form a stable equimolecular film even when the solution of the penetrating substance alone only forms Gibbs adsorbed layers and does not exist in the form of a coherent film When there is weak

association compression of the film forming substance can completely inhibit penetration

The experiments quoted in this paper were repeated with different apparatus and different samples of the same substance. The saponin from different sources showed small variations in its activity on the films mentioned.

#### SUMMARY

It is shown that molecular complexes can be formed by the injection of suitable organic compounds underneath various film forming materials. Two distinct types of complexes can be identified: those formed by association of a submerged polar group in the film forming material with a polar group in the material injected, and those in which a subsequent penetration of the hydrophobic portion of the film by the hydrophobic portion of material injected takes place. This subsequent stage is termed film penetration, and the stability of the resulting mixed film is shown to be due to molecular association. The extent of the molecular association is found to be dependent on the mutual interaction both of the polar and of the hydrophobic portions of the interacting molecules. Slight variation in the properties of either of these portions is found to exert a profound effect on the stability of the complexes. A remarkable degree of specificity is to be observed in complexes formed in this way.

Those molecules which show strong association in films make complexes in solution which can be distinguished by various methods such as inhibition of haemolysis and dispersion phenomena.

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## Molecular Interaction in Monolayers

### II—The Action of Haemolytic and Agglutinating Agents on Lipo-protein Monolayers

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In Schulman and Rideal (1937) several examples were given of the sensitivity in the extent of association in molecular interaction between molecules containing both polar and non-polar groups to slight alterations in the reactivities of either of these portions

In this paper an account is given of the interaction between organic molecules and protein monolayers and of the properties of these synthetic systems. It will be shown that they suggest possible mechanisms for the biological processes of lysis, agglutination, and sensitization. Two types of complex formation between molecules injected into the substrate and the molecules forming the monolayer have been established, those in which mutual association takes place by means of their polar groups alone, and those, the penetrating molecules, in which association is the result of the mutual interaction both of the polar and non-polar portions of the molecules.

Since the latter, the penetrating substance, must contain a relatively large hydrophobic portion in their molecular structure, they are all capillary active and in suitable concentration form colloidal solutions containing micellar aggregates. All such substances penetrate films of cholesterol and lyse red cells, and with the exception of saponin disperse protein films.

Substances which form complexes by interaction of their polar groups alone are of particular interest, for it is found that if macromolecules or polymers are formed of the reacting substance the reactivity of the macromolecule is very much greater than that of the single monomer. Since the macromolecule consists of a number of repeating units, each with its own reactive groups, it appears that the large macromolecule adheres to the reactive groups of the monolayer by more than one point of contact. Clearly, the question of appropriate spacing must enter into the extent of the number of points of contact of the macromolecule with the molecules forming the monolayer.

These macromolecular substances do not affect the surface tension or potential of water to a marked extent, are strongly adsorbed by protein films, in that they affect the phase boundary potential of these films, but do not increase the surface pressure. Thus, in contrast to the penetrating molecules they do not lyse but only sensitize and agglutinate red cells.

#### EXPERIMENTAL METHOD

The phase boundary potentials and changes in surface pressures were measured as described in Schulman and Rideal (1933). The protein chosen for investigation was a purified sample of the wheat protein gliadin, since not only is its composition relatively simple, containing at least 43 % glutamic acid, but it also possesses the valuable property of being soluble in 80 % alcohol, permitting it to be spread mixed with lipoids such as cholesterol to form homogeneous mixed films on aqueous substrates.

#### LIPID-PROTEIN FILMS

In fig. 1 are shown the characteristics of a film formed by dispersion of an 80 % alcohol-water solution containing cholesterol and gliadin in ratio of 1 : 4 in contrast with a film of gliadin alone on to a substrate of  $M/25$  phosphate buffer at  $pH\ 7.2$ . Gliadin in 80 % alcohol-water solution denatures and becomes insoluble quickly, consequently results have to be taken with fresh solutions.

An experimental error in calculating the quantity of protein in the solution is therefore always liable to occur, but the results obtained agree fairly well with those given by Hughes and Rideal (1932), who spread the gliadin films from the solid protein. Their results have been confirmed by another method (private communications by Mr J. S. Mitchell).

The behaviour of the mixed film on compression is of interest, for whereas the pure protein film gradually sets to a tenacious gel before crumpling, the lipid-protein film is first of all liquid and on compression sets to a gel. On further increase of the pressure in the region where the protein film collapses into long striations the lipid-protein film suddenly and sharply liquefies and on continued compression acquires all the characteristics of a film of cholesterol. Ultimately, the film exhibits a phase boundary potential (380 mV) identical with that of the cholesterol at the same area, viz.  $40\ A^2/mol$ , the area being based on the assumption that all the gliadin has been driven from the surface. In the dark field ultra-microscope the well-known "punkt struktur" of Zocher and Staebel exhibited by films of

cholesterol can be observed to appear in these compressed lipo protein films

On expansion and recompression the whole cycle is found to be reversible and this process can be repeated several times before the regelation on expansion becomes less marked. It is interesting in this connexion that on leaving the films standing for periods of an hour the slope of the compression curve becomes very much steeper such that now the pressure for the ejection of the protein by the cholesterol rises from 22.5 dynes to 27 dynes at the same surface density approximately  $4.7 \times 10^{-7} \text{ g/cm}^2$ . The initial sharp rise starting from a pressure of 1.5 dynes at  $1.25 \times 10^{-7} \text{ g/cm}^2$  remains the same with time. The weight per sq. cm. is calculated on the combined weights of the cholesterol and gliadin in the alcohol solution and they were spread by the Gorter method.

The surface potential curve shows a value of 200 mV at this point and increases sharply to 300 mV at  $2.0 \times 10^{-7} \text{ g/cm}^2$  when gelation of the film sets in and the curve slopes over to a value of 345 mV at  $4.8 \times 10^{-7} \text{ g/cm}^2$ . At this point the film suddenly liquefies and the potential rises to 380 mV at  $7.75 \times 10^{-7} \text{ g/cm}^2$  where it collapses. This value is approximately  $40 \text{ \AA}^2$  calculated on the cholesterol content of the solution and where the cholesterol film normally collapses. The collapsed films do not reveal any of the usual long striations of gliadin observable in the dark field ultra microscope.

As with mixed films of long chain alcohols and oleic acid at pH 2 the association complex between cholesterol and the protein is too weak to withstand a compression greater than 25 dynes/cm. and the protein like the oleic acid is forced beneath the film. The lipo protein differs from the former mixed film in that on release of the pressure the protein reappears to form a uniform mixed film immediately whilst the displaced oleic acid only gradually re enters the film of alcohol. It must be inferred that the protein is retained underneath the cholesterol film and attached to it at appropriate points so that the compressed lipo protein film must be regarded as a duplex film with cholesterol on one side attached to a gliadin monolayer on the other.

This gelation and liquefaction of the lipo protein is very sensitive to environment and ratio of lipid protein concentration. On pH's more acid than 7 (the isoelectric point of the protein) no gelation of the mixed film of 20% lipid takes place. Likewise increasing the lipid content results in non gelation over the whole range of pH. The cholesterol thus interacts with protein on both sides of its isoelectric point in a different manner. It has been shown to interact with both carboxyl and amino groups separately.

in the fatty acids and psychosine penetrations (Schulman and Rideal 1937)

The degree of interaction of the OH group varying according to the ionization of the acid and amine group

#### ADSORPTION ON PROTEIN FILMS

The experiments quoted in this paper on the action of soaps and soap protein complexes at various pH's on protein films have been taken from those already described in Parts I, II and III of Schulman and Rideal (1933) and Schulman and Hughes (1935)

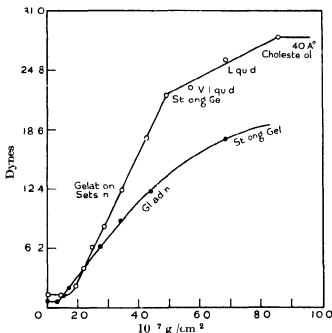


FIG 1—Mixed cholesterol protein film 20% cholesterol 80% gliadin pH 7.2

In earlier communications it was shown that when long chain fatty acids at pH 7.2 were injected underneath a film of protein e.g. albumen casein or gliadin the latter was rapidly displaced resulting in the formation of a monolayer of fatty acid the protein being carried into solution as a lipo protein complex

In contrast to the soaps if a solution of saponin containing 5 mg in 300 c.c. at pH 7.2 be injected underneath a film of gliadin there is a rapid

change in the phase boundary potential of 70 mV from 330 to 260 mV but only a small and slow change in the surface pressure some 6–10 dynes/cm in an hour (The protein being placed on the surface at 5 dynes/cm compression) As will be described later this observation suggests that the lytic properties of saponin on red cells are determined by its reaction with the cholesterol rather than the protein

The action of the long chain sulphate on a protein film is very similar to that of the carboxylic fatty acids in that there is a rapid displacement of the protein at concentrations as low as  $10^{-4}\%$ . The sulphate however is too soluble to form stable continuous films but the solution of this concentration possesses a surface tension of 12 dynes/cm lower than that of water. That the lipo protein is also capillary active is shown by the fact that the solution after injection of the sulphate and apparent disappearance of the protein film has a surface tension of 19 dynes/cm lower than that of water

In this connexion the action of lytic agents on the mixed lipo protein is interesting. The soaps disperse the films very readily and one is unable to make stable lipo protein films containing a fatty acid at neutral pH's. On the other hand saponin which does not displace proteins but is adsorbed on to the films behaves differently

At low compressions before the cholesterol separates from the protein (fig 1) the penetration of the protein film by saponin is greatly aided by the presence of the cholesterol. On the compressed film where the cholesterol is on the surface with the protein underneath no or only very slow penetration of the cholesterol film takes place. The protein apparently adsorbs the saponin and prevents it attacking the cholesterol above it

To demonstrate this the following experiments were carried out

5 mg of saponin were injected into the 300 c.c. of underlying solution at pH 7.2 under the following films

- (1) Gliadin film at 5 dynes compression (gel)
- (2) 20% cholesterol gliadin film at 5 dynes compression (liquid)
- (3) 20% cholesterol gliadin film at 22.5 dynes compression (liquid)
- (4) Cholesterol alone at 22.5 dynes compression (liquid)

On (1) the surface pressure rose 10 dynes in 30 min. and the film remained a strong gel

On (2) the pressure rose 20 dynes in 30 min. and the film changed from a liquid to a strong gel

On (3) there was only a small change in pressure of some 8–10 dynes in 30 min. compared with (4) at the same starting pressure where there was an instantaneous increase of pressure of some 30 dynes in a few minutes

### THE ACTION OF AGGLUTINATING AND SENSITIZING AGENTS ON PROTEIN FILMS

The difference in behaviour of gallic and tannic acids when injected under a film of gliadin presents some interesting features. In fig 2 is shown the adsorption of these substances by a film of gliadin at pH 7.2. A concentration of  $1.66 \times 10^{-3}\%$  of tannic acid reduced the phase boundary potential of the gliadin film from 330 to 230 mV ca 100 mV in a few minutes and

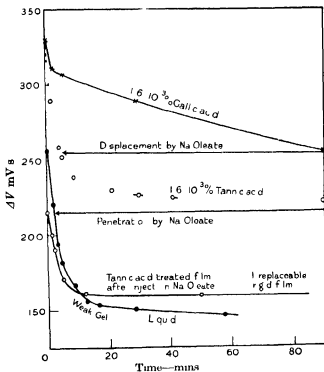


FIG. 2

reduced the surface pressure of the film from 5 dynes/cm to 3 dynes/cm. The film has thus become slightly more hydrophobic to the underlying solution as a result of the adsorption of the tannic acid. With solutions of gallic acid of the same weight concentrations in spite of its greater rate of diffusion the surface potential of the gliadin film is reduced only by 100 mV in 3 hr. In tannic acid there are ten gallic acid units and the effect of conversion of the molecule into a macromolecular polymer is clearly marked.



Neither gallic nor tannic acids in these concentrations affect the surface tension or potential of a free water surface to a detectable extent

After the adsorption of the tannic and gallic acids by the protein was completed (fig 2) 2.5 mg of sodium oleate were injected into the 300 c.c. of underlying solution. This quantity of sodium oleate when injected under a film of protein very rapidly peptizes and disperses the film leaving as we have noted a monolayer of the soap at the interface (Schulman and Hughes 1935). When injected under the gallic acid treated film it is found that a slow dispersion of the film occurs the process being complete in about 20 min. With the tannic acid treated film however although penetration takes place no dispersion occurs and the film remains a strong rigid solid possessing a somewhat higher phase boundary potential than that given by the surface formed by injection of the oleate underneath an untreated protein film. It would appear that the protein units in the surface have been sufficiently linked together by reaction with the galloyl units constituting the tannic acid macromolecule to render them non dispersible.

That such a non dispersible network can be formed is shown by the effects of injection of 200 mg sodium silicate into the underlying solution at pH 6.5. The silicic acid formed by hydrolysis is slowly adsorbed by the protein film as revealed by a fall in phase boundary potential of 40 mV of the ghadin film of 350 mV in 2 hr. Upon injection of 2.5 mg of sodium oleate as before the film undergoes dispersion but if the silicate be left in contact with the protein film for 12 hr before injection of the sodium oleate the film is found to be non dispersible although penetration of the soap into the film occurs. The silicic acid undergoes slow polymerization in solution and the polymeric network links the constituents of the protein film together as with tannic acid.

The potential of the ghadin silicic acid film after penetration by the sodium oleate is found to be as the tannic acid treated film some 20 mV higher than that of the simple lipo protein system at pH 6.5.

Sensitizing dyes such as Janus green are strongly adsorbed by protein films  $1.6 \times 10^{-3}\%$  in the underlying solution increasing the phase boundary potential of the ghadin film of 330 mV by some 160 mV in an hour at pH 7.2. No large change in surface pressure is detectable at these concentrations although at concentrations of  $5 \times 10^{-3}\%$  an increase of 4.5 dynes/cm is obtained in 3 hr. Since the dye is not macromolecular in character the resulting protein dye film is readily dispersible by sodium oleate.

## A POSSIBLE MECHANISM FOR THE LYSIS OF RED CELLS

A number of different views have been expressed as to the structure of the membrane of the red cell (Ponder 1936). The view has frequently been advanced that there is an actual monomolecular membrane enclosing the cell contents although this membrane may in part be linked across so as to ensure the maintenance of the characteristic structure of the cell. Calculations based upon electrical capacity measurements although they must be accepted with reserve likewise suggest that the superficial layer has a thickness of the order of a monolayer (Fricke 1925). The analytical data of Jorpes (1932) suggest that the composition of the membrane is *ca* 80% of a water insoluble protein of isoelectric point *pH* 5.5 and 20% of lipid. The association of cholesterol and gliadin to form remarkably stable lipo protein monolayers suggests the view that the membrane of the red cell may consist of a similar mixed film.

It is easy to visualize the action of capillary active materials which possess the property of penetrating lipid films and dispersing protein films on such a membrane. Thus we might anticipate that saponin for example which in concentrations of 10<sup>-3</sup>% rapidly penetrates films of cholesterol increasing the surface pressure some 50 dynes/cm but is only adsorbed by monolayers of gliadin would exercise its lytic activity primarily on the lipid portion of the membrane of the cell and only secondarily on the protein constituent. This is further supported by the increased penetration of a cholesterol gliadin mixed film at low compression by saponin in contrast to a film of the protein alone.

Furthermore saponin should form complexes with any cholesterol and protein available in the plasma and thus become inactive for lysis. This view of the action of saponin is further supported by its action on various bacteria examined by Klein and Stone (1931) where lysis could be effected by prior treatment or sensitizing of the bacteria with cholesterol. If the surface of the bacteria contains protein we have noted that the cholesterol would interact with the protein and thus enable the saponin to penetrate the membrane through the lipo protein complex.

The lytic action of fatty acids taurocholic acid and psychosine should be even more radical than that of saponin in that not only do these substances penetrate and rapidly form complexes with films of cholesterol or of sphingomyelin (the surface pressure rising some 50 dynes/cm from 10 dynes/cm film pressure within 2 min. on injection of concentrates of *ca* 10<sup>-4</sup>%) but at the same time they can disperse and disintegrate insoluble monolayers of proteins.

Support to the view that such dispersion does in fact take place is found on examination of the structure of the red cell ghosts formed on lysis, with saponin the ghosts are well defined but with fatty acids confirming Plattner and Hintner (1930) definite signs of disintegration are observed.

Finally, we note that the ease of penetration and dispersion by fatty acids of protein monolayers is greatly affected by the pH (Schulman and Hughes 1935). At pH 5.5 the rate is relatively slow, thus washed blood cells are not lysed by small quantities (sublytic) of soaps at this pH although the soap is adsorbed. If small quantities of serum protein pH 7.3 be now added to the system the cell wall fatty acid complex is brought by the serum protein to the reaction of pH 7.3 and the soap both penetrates and disperses. At the same time if serum be added to the blood cell suspension before the addition of the soap—a large fraction of the fatty acid is rendered inactive by combination to form lipo proteins in the bulk phase. A film of protein can be protected from dispersion by fatty acids and ferments by first adding a large excess of protein into the underlying solution (Schulman and Rideal 1933). That this change in ease of penetration on serum sensitization is due to a change in pH of the cell wall proteins is supported by the observation that those proteins which definitely buffer on the acid side, e.g. gelatin and egg albumen of isoelectric point ca. pH 4.6, do not sensitize. According to Roy and Sen (1929) sensitization may be effected by the addition of amines or alkalis.

The ease of penetration of the fatty acids is found to run parallel with the lytic activity not only in respect to the similarity of their dependence on the pH but also on the length of chain of the fatty acid employed, thus whilst palmitic acid penetrates and disperses films with great rapidity (Schulman and Hughes 1935) and is extremely haemolytic, pelargonic acid penetrates but very slowly and is lytic only after long periods of time.

#### A POSSIBLE MECHANISM FOR AGGLUTINATION AND SENSITIZATION OF RED CELLS

We have noted that agglutinating agents such as tannic acid are strongly adsorbed when macromolecular by films of proteins. They do not affect films of fats, sterols, alcohols and esters. It is possible that the phenolic group in each unit of the macromolecule undergoes association with the keto imido linkage of the polypeptide chain (Kruyt and De Yong 1922).

The adsorption of excess tannic acid by a protein film effects the reduction of the surface pressure of such a film when compressed to ca. 5 dynes/cm to practically zero value. The mixed film no longer lowers the

surface tension of water and must be regarded as existing in the form of a skin. Such a behaviour is exhibited by casein which will not spread at its isoelectric point  $pH\ 4.6$  but if spread at a slightly more alkaline  $pH$  and the substrate be brought back to  $pH\ 4.6$  an insoluble inactive film of casein remains on the surface as a skin. In the same way the red cell membrane on adsorption of tannic acid may be regarded as rendering the cell water interface as hydrophobic and agglutination occurs provided that the electrokinetic potential of the now suspensoid system is not too high. Such tannin treated membranes still permit of the penetration of lytic substances (Reiner and Fischer 1929) such as the soaps but the ghosts should be resistant to disintegration since a tannin treated protein film is penetratable but not dispersible. Silicic acid should behave in a similar manner but as we have noted the process of polymerization of the orthosilicic acid to form a macromolecular hydrophobic polymer on the surface of the cell is a process which proceeds relatively slowly.

It is interesting to compare the film reactivities of sensitizing dyes such as Janus green with those of tannin and silicic acid.

In dilute solution Janus green is adsorbed by a protein film but does not appreciably affect the surface pressure. On increasing the bulk concentration however (up to  $15\text{ mg./300 c.c.}$ ) the dye raises the surface pressure by some  $4\text{ dynes/cm.}$  i.e. the dye commences to penetrate the film and one might anticipate a lytic activity in high concentrations (Browning and Mackie 1914, Ponder 1928). The dye in this respect thus presents certain analogies to the fatty acids and the effects of adding serum to cell suspensions containing sublytic concentrations of dye present other points of agreement. The sensitization of red cells to sublytic concentrations of fatty acids was described in the last section.

From these considerations we may regard the coating of a red cell by the antibody globulin in sensitization as fulfilling a number of separate and important functions. In the first place it renders the system relatively hydrophobic the complex behaving as a skin rather than as a film. Again it changes the  $pH$  of membrane from  $ca.\ 5.5$  to one more alkaline and finally the cell is coated with a sheath of denatured protein.

We have seen that fatty acids are strongly adsorbed out of a protein solution by any film of denatured protein and that their penetration is practically completely stopped by change of  $pH$  to the acid side of  $pH\ 7$  the optimum lying between  $pH\ 7$  and  $8$  (Schulman and Rideal 1933, Schulman and Hughes 1935).

It is not difficult to visualize a system whereby a lytic substance which lowers the surface tension and penetrates films is present as a complex

with cholesterol in the serum and as such does not exert its lytic activity but if on sensitization the red cells are coated with a sheath of denatured globulin of pH 6.8 (Felton 1932) the lytic substance separates from its carrier and is adsorbed by the sensitized cell which it subsequently penetrates. Such a system would present many analogies to the observed operation of the complement reaction especially as the lipid protein complexes are apparently very sensitive to environment i.e. pH and salt concentration.

It is by no means improbable that the final pH of the protein coated cell wall is dependent on the concentration of electrolytes in the surrounding medium since below a certain ionic strength the effect of electrolytes at constant pH on the electrokinetic potential is not in agreement (Kemp and Rideal 1935) with the theory of Debye-Hückel and Henry owing to the intrusion of a Donnan distribution of hydrogen ions.

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#### SUMMARY

The properties and behaviour of lipo-protein films of cholesterol and ghaadin are described and the results of injection of lytic agglutinating and sensitizing agents beneath such films are examined.

It is shown that all lytic agents examined either penetrate cholesterol films or disperse protein films and are all capillary active. Agglutinating or sensitizing agents only adsorb on protein films and show no penetration properties and no appreciable capillary activity.

The size of the reacting molecules is shown to be important for all these phenomena. From these experiments on lipo-protein films tentative conclusions are drawn concerning possible mechanism of lysis, agglutination and sensitization of red cells.

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591 69 595 792 *Trichogramma*

## The Sense used by *Trichogramma* to distinguish between Parasitized and Unparasitized Hosts

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### INTRODUCTION

It was demonstrated in an earlier paper of this series (Salt 1934 *b*) that the chalcid parasitoid *Trichogramma evanescens* is able to distinguish healthy hosts from those already parasitized. The present study is an attempt to answer the question, "By what sense is that discrimination performed?"

The material and methods used in these investigations have been fully described before (Salt 1934 *a*) and, if it is recalled that *Trichogramma* is a minute parasitoid developing in the eggs of insects, especially moths, no further explanation is necessary for the understanding of this paper

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## SIGHT, HEARING, AND TOUCH

Sight is easily ruled out of the discussion by experiments showing that the discrimination is exercised in complete darkness. Twenty-five eggs of the grain moth, *Sitotroga cerealella*, were fastened on graph paper,  $\frac{1}{8}$  in. apart, in a square that could be covered by a small glass container  $\frac{3}{4}$  in. across and  $\frac{1}{4}$  in. deep. One female *Trichogramma* was put with them and the dish was then placed in a light-tight container in an incubator at 25° C for 8 hr. At the end of that time the parasite was removed and the host eggs were examined microscopically for eggs of the parasite. The experiment was repeated four times, with the results shown in Table I.

TABLE I.—RESULT OF EXPOSING 25 EGGS OF *SITOTROGA* TO ONE FEMALE *TRICHOGRAMMA* FOR 8 HR. IN COMPLETE DARKNESS

Exp	Number of hosts containing				
	0 egg	1 egg	2 eggs	3 eggs	4 eggs
1	7	18	.	.	.
2	4	21	.	.	.
3	3	22	.	.	.
4	1	24	.	.	.
Obs total	15	85	0	0	0
Cal total on hypothesis of random distribution	43	37	15	4	1

In the four experiments, 85 parasite eggs were distributed among 100 hosts. Using the formula

$$z = N^x C_p \left( \frac{1}{N} \right)^p \left( 1 - \frac{1}{N} \right)^{x-p},$$

where  $N$  represents the number of hosts,  $x$  the number of parasites distributed, and  $z$  the number of hosts containing  $p$  parasites, it can be shown that if the distribution had been completely random, 43 of the hosts would have remained unparasitized, 37 would have contained one parasite, 15 two, 4 three, and 1 four parasites. In fact, however, 15 remained unparasitized and 85 contained one parasite. In no case was a host parasitized more than once. The discrimination was perfect. We may conclude, since the parasitization took place in complete darkness, that sight is not the sense used by *Trichogramma* in distinguishing healthy from parasitized hosts.

The only sounds that might distinguish healthy and parasitized hosts are (1) sounds produced by the embryo itself; (2) the different sounds

produced by healthy and parasitized hosts when palpated by the antennae or feet of the parasite. The first alternative is clearly untenable because hosts in which the embryo is still undeveloped or is dead and therefore could not itself make any sound are attacked. The second alternative is very unlikely since if different sounds arise from the palpation of healthy and parasitized hosts far more different sounds must come from the great range of hosts attacked not only different species of insect eggs but also plant seeds globules of glass and many other kinds of false hosts (Salt 1935). It is then extremely unlikely that the parasite hears a difference between healthy and parasitized hosts.

The sense of touch might enable a parasite examining a parasitized host to feel the hole or scar where the chorion had been perforated. To investigate this possibility 25 eggs of *Sitotroga* were exposed to a parasite under observation. Each time the parasite withdrew from a host the exact spot at which the ovipositor had been inserted was marked on the host with a minute dot of lamp black and water. Forty healthy eggs from the same lot were marked arbitrarily with a similar black dot. Thirteen of the parasitized hosts were now fastened in squares 1 3 5 12 14 etc. of the graph paper with the black spot down. The hole made by the ovipositor therefore was glued to the surface of the graph paper and could not be felt by a parasite coming later. The intervening spaces 2 4 11 13 etc. were filled with 12 healthy hosts that had been touched with black their black spot also down. A second parasite was now placed on this group of 25 hosts and kept under continuous observation. Each time it attacked a healthy host the latter was removed and its place filled by another healthy host each time it attacked a parasitized host that was replaced by a new parasitized host always with the black spot marking the point of parasitization in contact with the substratum. The substitution was easily carried out while the parasite was engaged with its next host for *Trichogramma* is not easily disturbed while it is ovipositing and a host can be removed and replaced even immediately beside it without disturbing the parasite or interrupting the experiment. Twenty five attacks were observed and continuous notes were made of every host touched examined or attacked. The experiment was repeated four times twice with 13 parasitized and 12 healthy hosts and twice with 12 parasitized and 13 healthy hosts.

In each of three experiments 5 and in the fourth experiment 7 of the eggs already parasitized were selected and attacked by the second female. Of the 100 attacks therefore 22 were on hosts already parasitized and 78 on healthy hosts.

Since there were equal numbers of parasitized and unparasitized hosts



equally available to the second parasite, if it had been unable to distinguish the two kinds of hosts, equal numbers of them should have been attacked. Actually, 78 attacks were made on the healthy hosts and only 22 on the parasitized. It is clear that to a considerable extent the parasite was able to distinguish between them. The numerical result is supported by other evidence. In the course of the experiment it was observed that the parasite frequently rejected the upturned parasitized hosts but eagerly attacked the next healthy host it found. Moreover, of the 22 attacks on parasitized hosts, 14 were of abnormally short duration (cf Salt 1934 b, p 464). Finally, of the 22 hosts apparently superparasitized, only 4 were found on microscopic examination actually to contain two parasite eggs, the other 18 contained only one each.

It may be concluded, then, that *Trichogramma* is able to distinguish parasitized from unparasitized hosts even when the hole made by the ovipositor of a former female is put out of reach by overturning the host. The number of mistakes is indeed, greater than when the eggs are left in their original position (cf data in Salt 1934 b, p 466), but this, as will be clearly shown below, is due to another factor.

The possibility that an ovipositing female leaves on the surface of its host a sticky substance which is felt by the muscle sense of another parasite, can be dismissed as follows. The preceding experiment shows that if parasitized hosts are distinguished by stickiness, that condition must be perceptible over a considerable part of the surface of the host, away from the oviposition hole. But a sticky substance present in such minute amount would readily dry and, to be felt as sticky, would have to be redissolved by the second parasite, which would therefore have to provide the solvent. Now the sticky substance would have to be water-soluble, because experiments described below show that what distinguishes parasitized hosts can readily be washed off with water. But hosts painted with water soluble gums, gum tragacanth and gum arabic, in several concentrations up to 20% by weight, are not in the least avoided by *Trichogramma* as soon as they are dry. It appears very improbable, then, that parasitized hosts are distinguished by being sticky.

The several experiments and observations detailed in this section rule out the senses of sight, hearing, and touch, or, at least, make it very unlikely that these senses are concerned in the discrimination. By this process of elimination, attention is guided to the chemical sense of taste-smell which, as will be shown, gives a definite and positive result.

## THE CHEMICAL SENSE

Since *Trichogramma* accepts and attacks true hosts of many different species as well as several kinds of false hosts it is unlikely that acceptable hosts are distinguished by an attractive odour which they suddenly lose on being parasitized. It is much more likely that they lack a deterrent odour which is imparted to them when they are parasitized. Search is first to be made, then, for an inhibitory smell peculiar to parasitized hosts.

When an egg of *Sitotroga* is perforated by the ovipositor of the parasite, a small quantity of the fluid contents of the host sometimes oozes out of the hole. It might be supposed that a second parasite could smell those contents and so recognize the host as one previously parasitized.

To investigate this possibility 25 eggs of *Sitotroga* were fastened  $\frac{1}{8}$  in apart in  $\frac{1}{4}$  sq in. of a graph paper disk. A number of fresh *Sitotroga* eggs were crushed in a small mortar and droplets of their contents were applied with a very fine brush to the surface of the 13 hosts placed in squares 1, 3, 5, 12, etc., of the graph paper. The 12 hosts in the intervening squares were touched with another fine brush carrying only clean water. A parasite was placed with the 25 hosts and allowed to attack them under observation as in the experiments described above. When a host had been attacked it was removed and replaced by another host which was then touched with egg contents or with water as required. By means of this substitution technique, the parasite had always 13 of one kind and 12 of the other kind of host available to it. Each experiment was continued until 25 attacks had been made. Four experiments were performed, two with 13 treated and 12 wetted hosts and two with the reverse. The results are shown in Table II.

TABLE II.—SELECTION OF HOSTS TREATED WITH CONTENTS OF CRUSHED *SITOTROGA* EGGS AND HOSTS TOUCHED WITH WATER

Exp	Hosts touched with egg contents	Hosts touched with water
1	14	11
2	13	12
3	13	12
4	10	15
Total	50	50

Exactly equal numbers of attacks were made on the treated hosts and on the hosts touched merely with water. The attacks on both groups of hosts were of normal duration and in most cases gave rise to adult parasites.

in the next generation. From this result it is clear that *Trichogramma* does not avoid hosts bearing the smell of their exuded contents and therefore cannot be said to reject parasitized hosts on that account.

It appears that the deterrent odour must be one supplied by the parasite, not by the host. A smell might be left by *Trichogramma* (1) as it walks on the host in the process of examination, (2) as it stings the host, (3) by laying an egg in the host. In the first case the feet of the parasite, in the second the ovipositor or its glands, in the third the parasite egg itself, might be the source of the odour. Since each of these three activities includes that which precedes it, analysis must commence with the first.

To determine whether a second parasite could distinguish hosts which had been merely walked upon but neither stung nor oviposited in by another parasite, 13 *Sitotroga* eggs were fastened in squares 1, 3, 5, 12, etc., of the graph paper and a female *Trichogramma* was introduced. The parasite was allowed to examine each of the eggs in turn which it did in the manner normally preceding oviposition but just as it accepted an egg and was about to unsheath its ovipositor, it was struck aside with a fine brush. The duration of the examination was measured with a stop watch. When each of the 13 hosts had been walked on in examination for at least 30 sec., the female was removed. In the intervening spaces 2, 4, 11, 13, etc., were now placed 12 fresh eggs. A second female was introduced and allowed to select 25 hosts for parasitization. When one of the fresh eggs was selected, the parasite was allowed to consummate the attack and, when it had moved off that host was replaced by another fresh one. In the few cases when the parasite accepted a 'dirty' host one of the 13 walked on by the original female, it was pushed aside when it raised its abdomen to oviposit but the acceptance was counted as an oviposition. With this technique, the parasite always had about it 12 fresh hosts and 13 hosts which had been walked upon but not parasitized or stung. Running notes were kept as in all these experiments of every egg touched, examined or attacked, and of the time and duration of each attack. Since this turned out to be the crucial experiment of the investigation it was performed eight times, giving a total of 200 attacks. In four experiments there were 13 "dirty" and 12 fresh hosts, and in the other four there were 12 "dirty" and 13 fresh hosts. The results are given in Table III.

Observations in the course of these experiments showed conclusively that the parasite was able to distinguish between clean eggs and those that had previously been walked upon by another female. The 'dirty' eggs were approached and touched and yet, in nearly every case, were immediately rejected, while, in the first part of the experiment at least,

when the parasite had still plenty of eggs to deposit, the next "clean" host encountered was accepted and attacked. The numerical result leaves no room for doubt of this discriminative ability. The numbers of contacts with "clean" and with 'dirty' eggs were practically equal, and yet the number of "clean" eggs accepted and parasitized was 172, the number of 'dirty' eggs only 28.

TABLE III—SELECTION OF CLEAN HOSTS AND HOSTS PREVIOUSLY WALKED ON BY ANOTHER PARASITE

Exp	Number of contacts		Contacts with eggs already parasitized	Acceptances		Rejections	
	With clean eggs	With dirty eggs		Of clean eggs	Of dirty eggs	Of clean eggs	Of dirty eggs
1	34	28	4	25	0	5	28
2	43	41	3	24	1	16	40
3	44	42	8	22	3	14	39
4	41	42	3	22	3	16	39
5	49	52	6	22	3	21	49
6	26	27	4	20	5	2	22
7	46	51	15	20	5	17	40
8	54	46	7	17	8	30	38
Total	337	329	50	172	28	121	295

From this series of experiments it can be concluded (1) that females of *Trichogramma* are able to distinguish between clean hosts and hosts that have previously been merely walked upon—not stung or oviposited in, by another female of their species—and (2) that they avoid attacking hosts that have been walked on just as they avoid attacking parasitized hosts.

This result explains a difficulty in a previous experiment. On p. 60 above, where the sense of touch was being investigated, it was found that, when parasitized hosts were turned upside down, the parasite made a greater number of mistakes than when the hosts were left in their original position. This is now readily understandable for when the hosts were overturned, a part of the surfaces on which the previous parasite had walked was hidden, and their formerly lower surfaces, on which the parasite had not walked, was exposed.

The foregoing experiment leads to the view that the means of discrimination is an external trace left by the parasite upon the surface of the host. This view can be put to the test. If the trace is external and is, as all the preceding experiments suggest, of a chemical nature, it should be possible to wash it off. The parasites should then no longer be able to discriminate

Experiments to test the hypothesis were performed as follows *Trichogramma* females were allowed to walk upon 30 *Sitotroga* eggs, as in the preceding experiment, for at least 30 sec, but not to sting or parasitize them. These 30 eggs were then washed with the tip of a soft brush and ample water. Thirty fresh eggs, never walked upon, were washed in a similar manner but with a different brush and in different water. Thirteen of the washed "dirty" eggs and 12 of the washed "clean" eggs were exposed to a parasite in the now familiar manner, and the eggs that were parasitized were replaced as usual. In order that no chemical trace should be transferred, three different brushes were used for the removal of the hosts attacked and the replacement of the two kinds of new hosts. The duration of each attack was measured with a stop watch, and the experiment was continued until 25 attacks had been made. Two experiments were performed, with the results shown in Table IV.

TABLE IV—RESULT OF EXPOSING WASHED "DIRTY" AND WASHED "CLEAN" HOSTS TO *TRICHOGRAMMA*

	Exp	Number attacked	Average duration of attack min sec	Number actually parasitized
Washed dirty hosts	A	11	2 17	11
	B	13	3 24	13
	Total	24	2 53	24
Washed clean hosts	A	14	2 29	14
	B	12	3 9	12
	Total	26	2 46	26

Practically equal numbers of the washed "dirty" and the washed "clean" hosts were attacked. The duration of the attacks on the two kinds of hosts was the same. The two kinds of hosts were actually parasitized and produced nearly equal numbers of adult parasites in the next generation. The parasites, then, did not in any way discriminate between the washed "dirty" hosts and the washed "clean" hosts.

It can be concluded that washing in water completely removed the means by which, in the preceding experiment, the parasite was able to discriminate between clean hosts and hosts that had been walked upon by another parasite. The trace, then, is certainly external.

The fact that the means of discrimination can be removed with water suggests, but does not prove, that the trace left by the parasite on the host is chemical in nature. The physical disturbance of washing might remove indications other than chemical traces from the surface of the host.

Only a chemical substance however could be collected in solution and used artificially to protect clean hosts from attack as was done in the following experiment

Two hundred females of *Trichogramma* were put into a small clean vial and allowed to run about on the glass for 2 hr They were then knocked out of the vial and the glass on which they had walked was washed with a clean brush and a very small drop of water Fifty hosts of which 25 were smeared with this solution and the alternating 25 merely wetted with fresh water were exposed to a female parasite for 4 hr Fifteen dishes were used In 5 dishes the solution was used at full strength in 5 it was slightly diluted with a small drop of water and in the last 5 dishes it was diluted still more with 2 drops of water The results are shown in Table V

TABLE V.—SELECTION OF HOSTS SMEARED WITH SOLUTION FROM A SURFACE THAT HAS BEEN WALKED UPON BY *TRICHOGRAMMA* AND OF HOSTS WETTED WITH WATER

	Dish	Hosts smeared with solution	Hosts wetted with water	Total number of hosts parasitized
Solution at full strength	1	1	13	14
	2	1	18	19
	3	0	21	21
	4	2	19	21
	5	1	22	23
	Total	5	93	98
Solution at first dilution	1	1	14	15
	2	4	11	15
	3	2	16	18
	4	5	14	19
	5	3	18	21
	Total	15	73	88
Solution at second dilution	1	5	4	9
	2	7	9	16
	3	9	13	22
	4	11	11	22
	5	8	16	24
	Total	40	53	93

It is clear from the numerical results that the parasites avoided the hosts that had been smeared with the solution and further that the discrimination decreased as the solution was diluted The fact which emerges from this experiment that the deterrent substance is left by the parasite not only upon hosts but also upon the general surface on which it walks will

be discussed later. The point of interest here is that the substance can be collected in aqueous solution and applied to clean, healthy hosts, which are thereby protected from attack.

It is to be concluded that the trace which is left by females of *Trichogramma* upon surfaces on which they have walked, and which is recognized by other parasites that follow them, is chemical in nature.

Two lines of investigation have converged to meet and agree at this point of the argument. A process of elimination, followed in the first part of this paper, indicated that the chemical sense was the one used in the discrimination. A direct study of the trace left by the parasite upon the host, pursued in the present section, shows that trace to be chemical in nature.

From all the preceding experiments the general conclusion can now be drawn that *Trichogramma* distinguishes clean hosts from those that have been merely walked upon by another female (and, so much the more, from those that have been parasitized) by means of its sense of taste-smell, which perceives a chemical trace left by the parasite on the surface of a host upon which it has walked.

#### THE SECOND DISCRIMINATIVE SENSE

In the two experiments summarized in Table IV, hosts that had been merely walked upon by *Trichogramma*, not attacked or oviposited in, were washed and then, together with an equal number of clean hosts, were exposed to a parasite. That parasite accepted them and parasitized them without any discrimination whatsoever, whether of selection, of duration of attack, or of effectiveness of the parasitism. The question naturally arises, "What would have been the result if those hosts had been not merely 'dirty' but actually parasitized?" That question is answered by the following experiments.

Females of *Trichogramma* were allowed to parasitize 30 eggs of *Sitotroga*, which were then washed with a soft brush and ample water. Healthy eggs from the same lot were washed in a similar manner but with a different brush and in fresh water. Thirteen washed parasitized and 12 washed healthy eggs were now exposed to a parasite, and substitution of those attacked was carried out in the usual way. The duration of each attack was measured with a stop-watch. The experiment, it will be noticed, was identical with that described above, with the single exception that hosts that had been actually parasitized were used instead of hosts that had been merely walked upon. The experiment was repeated four times, twice with

13 parasitized and 12 healthy hosts and twice with 12 parasitized and 13 healthy hosts. The results are given in table VI.

TABLE VI—RESULT OF EXPOSING WASHED PARASITIZED AND WASHED HEALTHY HOST TO *TRICHOGRAMMA*

	Exp	Number attacks	Average duration of attack		Number actually parasitized
			min	sec	
Washed parasitized hosts	A	11	1	23	1
	B	11	1	44	1
	C	13	1	25	0
	D	15	1	29	1
	Total	50	1	30	3
Washed healthy hosts	A	14	3	26	13
	B	14	3	34	13
	C	12	2	39	12
	D	10	3	2	10
	Total	50	3	13	48

Exactly equal numbers of attacks were made on the washed parasitized hosts and on the healthy hosts. This result corroborates that of the previous experiment and shows that the parasite was unable to discriminate externally between healthy hosts and parasitized hosts that had been washed.

But the sequel took quite a different course. In the former experiment the attacks on the two kinds of hosts were of the same duration. In the present experiment 46 of the attacks on the washed parasitized hosts were abnormally short. In all but 4 cases they were abruptly terminated immediately after the ovipositor had penetrated into the host. The parasite seemed to discover *after inserting its ovipositor* that the host was already parasitized and quickly withdrew its sting and left. On the average the 50 attacks on healthy hosts lasted 3 min 13 sec; the 50 attacks on washed parasitized hosts only 1 min 30 sec.

Moreover in the former experiment the two kinds of hosts were both actually parasitized. In the present experiment although the washed healthy eggs were actually oviposited in by the second female, the washed parasitized eggs were not. The 50 washed parasitized hosts that were attacked a second time were at once examined microscopically for parasite eggs. Only 3 of them contained two eggs of *Trichogramma*; the other 47 contained only the one laid in them by the original female.

From this series of experiments three conclusions can be drawn: (1) Washing removed the external traces of parasitization and the parasite



was no longer able to discriminate externally between the healthy and the parasitized hosts (2) Nevertheless, the parasite was still able to distinguish between healthy and parasitized hosts when it had inserted its ovipositor into them (3) There are, then, two different senses involved One enables the parasite to recognize an external trace and thereby inhibits attack, the other enables the parasite to distinguish an internal difference and inhibits oviposition

This result serves to explain an observation made in the course of experiments already published (Salt 1934 *b*, p 464) and also above (p 60) that, when *Trichogramma* makes a mistake and attacks a host already parasitized it usually withdraws its ovipositor prematurely and goes off without laying an egg In those cases clearly it was this second discriminative ability that came into play

Further, the result has the effect of invalidating an experiment described at the beginning of this paper In the first series of experiments in which sight was eliminated by the demonstration that superparasitism is avoided even in darkness it might have been that this second sense was being used The parasite might have gone about in the darkness attacking healthy and parasitized hosts indiscriminately but, by premature withdrawal, have avoided laying two eggs in one host Sight, in short, is not eliminated as the sense used in the external discrimination of hosts One further experiment, therefore is necessary

Twenty five eggs of *Stotroga* were fastened in alternate squares of a piece of graph paper in an area  $\frac{1}{16}$  in square, and a *Trichogramma* female was allowed to examine but not to attack them When all had been walked upon, the parasite was removed and 25 clean eggs from the same lot were placed in the intervening squares There were now 50 eggs present, all of which were unparasitized but of which 25 were 'clean' and 25 were 'dirty' A female parasite was introduced and the dish put immediately into the incubator in complete darkness After 4 hr the parasite was removed and the dish was returned to the incubator for the eggs to develop The parasitism was observed on the fifth and subsequent days Five experiments were performed with the results shown in Table VII

Since the 'clean' and the 'dirty' hosts were present in equal numbers and equally exposed to the parasite, if no external discrimination had occurred equal numbers of the two sorts of hosts should have been parasitized In fact, however, 82% of the hosts attacked were "clean" It is clear that *Trichogramma* was able to distinguish "clean" and "dirty" hosts in complete darkness Sight, therefore, is not necessary for the external discrimination

TABLE VII—RESULT OF EXPOSING 25 "CLEAN" AND 25 "DIRTY" HOSTS TO 1 FEMALE *TRICHOGRAMMA* FOR 4 HR. IN COMPLETE DARKNESS

Exp	Total number of hosts parasitized	Number of "clean" hosts parasitized	Number of "dirty" hosts parasitized
A	15	14	1
B	19	16	3
C	19	16	3
D	20	17	3
E	20	13	7
Total	93	76	17

To sum up *Trichogramma* is able to distinguish parasitized from healthy hosts by means of two faculties. The first is its sense of taste smell which perceives a chemical trace left on the surface of a host that has been parasitized or even merely walked upon by another female. The second enables the parasite to recognize a parasitized host even when the trace has been washed off, but only after inserting its own ovipositor into the host. The first faculty receives its stimulus from the exterior of the host and inhibits attack, the second receives its stimulus from the interior of the host after attack and inhibits oviposition. Both, of course, operate so as to avoid superparasitism.

#### ADDITIONAL OBSERVATIONS

The principal object of this investigation is already attained, but there are one or two subsidiary problems that come naturally to mind and that must be dealt with before the study can be considered complete.

*The evaporation of the external odour*—In each of the first four experiments summarized in Table III, the selection by the second parasite followed the walking of the first immediately, without any more pause than was necessary to place the clean eggs in the intervening squares. Each of the last four experiments was performed with an interval of 1–3 hr. between the two stages. It is noticeable that the discrimination was less perfect in the second group. This suggests that the chemical trace left by the parasite on the surface of the host is volatile.

To examine the matter more critically, an experiment was performed in which the "dirty" hosts were placed in the air current from an electric fan for  $2\frac{1}{2}$  hr. before the second part of the experiment began. This time 16 "clean" and 9 "dirty" hosts were attacked. Of the contacts with "clean" hosts, 59% led to oviposition, of those with "dirty" hosts, only 29%. The second parasite was still able to discriminate to some extent,

but the number of "dirty" hosts accepted was greater than in any previous experiment

It is clear that the chemical trace disappears from the hosts after a time, and so leaves the parasite without its means of external discrimination. Since the chemical substance is so volatile, it may be looked upon as an odour.

In connexion with the evaporation of the odour, it is important to remember that in the above experiment and in those of Table III, the original parasite was on the host for only about 30 sec, only one-sixth of the time it would have remained there if it had been allowed to proceed with its oviposition. The odour on parasitized hosts, on which the parasite has stood for about 3 min, persists much longer, as is shown by the following experiment. Fifty hosts actually parasitized by *Trichogramma* were placed in a large constant temperature room at 18° C and exposed to the air so that the odour upon them might evaporate. A number of healthy hosts from the same lot were placed with them but in a closed container. After 24 hr and again after 48 hr, experiments were performed in which 13 parasitized and 12 healthy hosts were exposed to a parasite and those attacked were replaced as usual. After 24 hr, 5 mistakes were made in 25 attacks, a greater proportion than when there was no such intervening period (cf Salt 1934 b, Table V). After 48 hr, 8 mistakes were made in 25 attacks. The external discrimination, then, persisted to some extent for 2 days, but became less perfect as time went on. The internal discrimination, however, remained unimpaired, and four of the five wrong attacks in the first experiment and all eight in the second were followed by premature withdrawal.

From this section it appears (1) that the chemical trace left by the parasite on the surface of the host gradually disappears in time and therefore can be called an odour, and (2) that the amount of the odour left by a parasite is roughly proportional to the length of time the parasite is on the host.

*The nature of the odour left on the host*—The question of the nature of the substance left by the parasite on the host ultimately requires a chemical answer, but, on account of the small size of the animal and the minute quantity of the substance distributed, no immediate chemical analysis is likely. Biological suggestions are afforded by the following experiments.

A large number, 1500–2000, of parasites were cooled to 8° C to render them immobile, and were then easily collected on a damp brush and put into a drop of water in a vial. They were thoroughly washed in this water, some of them probably being crushed in the process, for the fluid took on a pinkish tinge. Of 50 eggs of *Sitotroga*, arranged on the graph paper

1/2 in apart as usual half were smeared with this fluid. The alternate hosts were touched with fresh water on a clean brush. A parasite was put on the 50 hosts for 4 hr at 25° C. The experiment was repeated five times. A total of 109 hosts were parasitized: 55 of those smeared with the fluid and 54 of those merely wetted with water. There was then no avoidance of the hosts smeared with a fluid bearing the general body odour of the parasite.

On the other hand experiments described on p. 65 above show that if glass on which a number of parasites have walked is washed and the solution obtained is applied to healthy hosts, those hosts are avoided as though they were parasitized. This demonstrates that the parasite distributes an odour upon the general substratum on which it walks, will be corroborated and used by Dr J. Laing in a forthcoming work on the finding of hosts by parasites. Here it is to be noticed that the substance that inhibits attack, although not obtained by washing the parasites themselves, is readily obtainable from a surface on which they have walked.

Three parts of the parasite—its antennae, its feet, and its ovipositor—touch a host that it parasitizes and might be the source of the odour. The ovipositor has been ruled out by the experiments described above (p. 62) in which the parasite walked on hosts and tapped them with its antennae but was not allowed to touch them with its ovipositor. The antennae are largely ruled out by the experiment described on p. 65 because when walking on glass the parasite gives the surface only an occasional tap, not at all like the rapid palpation given to a prospective host. Although there is no definite proof, then, the whole trend of this investigation suggests that the feet of the parasite are the source of the deterrent odour.

From this section it appears that the chemical trace left by *Trichogramma* on hosts upon which it has walked is not the general body odour of the parasite but a more specific smell produced, probably, by glands on the feet, and distributed not only upon hosts but also upon the substratum on which the parasite walks.

*The organs of discrimination.* Discrimination between healthy and parasitized hosts is not exercised at a distance. The parasite invariably touches the host before rejecting it. This suggests that the chemical trace is perceived only by actual contact and the question arises: By what organ is the discrimination achieved?

The examination of the host for its general suitability continues for a considerable time, averaging about 30 sec. The discrimination between healthy and parasitized hosts, however, is almost immediate. If the egg is to be rejected on the grounds of its previous parasitization, the female

merely passes across it her examination, if it can be called that, lasting for 2 sec. at the most, and usually less.

In that brief examination, two parts of the parasite touch the host, the antennae and at least the prothoracic feet. The prevailing idea that the antennae of insects are the principal receptors of olfactory stimuli (cf. Marshall 1935) and the fact that the antennae of other Hymenoptera are known to possess a chemical sense (ants, Forel 1908, honey bee, von Frisch 1919) suggest the antennae as the organs of discrimination in *Trichogramma*. But this cannot be taken for granted. Contact receptors of chemical stimuli have been shown by Minnich (1921, 1926) to occur on the tarsi of Lepidoptera and Diptera, and close observation of the behaviour of *Trichogramma* seems to implicate its feet. The antennae touch the host first, but no observation has been made of a female that was actually seeking a host rejecting an egg after touching it with the antennae and before touching it with the tarsi. In all observations made with this point in mind, the antennae and the feet both touched the host before rejection occurred. In one case especially noted, the parasite palpated the host three or four times with the antennae while still standing on the substratum. It moved forward and put only the prothoracic feet on the host, after which it immediately backed and went away.

On this point no experimental evidence has been sought and the question is raised only so that it shall not be too readily assumed that the antennae are the organs concerned in the discrimination.

#### DISCUSSION

It is now possible to give a general description of the manner in which *Trichogramma* discriminates between healthy and parasitized hosts. Before parasitizing a host the female always examines it very carefully by walking forward and back and turning around and around upon it, tapping it with the antennae. In the course of that examination, the parasite leaves on the surface of the host an odour which can be perceived by another female coming later. By that odour the second parasite recognizes the host as one that has already been visited, and avoids attacking it again. If the external odour is removed, the second parasite is unable to exercise this means of perception, and attacks healthy and parasitized hosts indiscriminately. At this point, however, another faculty comes into play. After inserting its ovipositor into a parasitized host the female is again able to perceive that it is already parasitized and, quickly withdrawing its ovipositor, goes off without laying an egg.

The question posed at the beginning of this paper, "By what sense is the discrimination performed?" must, therefore, be answered in two parts. *Trichogramma* first distinguishes healthy from parasitized hosts by means of its sense of taste smell which perceives an odour left by a preceding parasite on the surface of the host. This failing the parasite uses a second means of perception, probably again a chemical sense, but this time connected with the ovipositor, which distinguishes between the contents of healthy and parasitized hosts.

This result had its first effect on the technique of this series of investigations. The discovery that *Trichogramma* perceives and avoids hosts merely walked upon and not parasitized was made in February 1934. Since that time all experiments have been performed with carefully washed glassware, new graph-paper disks, different brushes for the placing and removal of hosts, and several other precautions against contamination of the eggs or of the substratum by the odour of the parasites. It is obvious that in any critical work on the behaviour of *Trichogramma* the keenness of its discriminative ability must in future be kept in mind.

The result of the present investigation makes intelligible some previous observations that have hitherto remained obscure. It was shown in an earlier paper (Salt 1934 b) that *Collyria calcitrator* and *Ibalia leucospoides* are also, like *Trichogramma*, able to avoid superparasitism. Unlike *Trichogramma*, however, these parasites cannot reach their host to examine it with their antennae, *Collyria* because its host, *Cephus pygmaeus*, is inside a wheat stem, *Ibalia* because its host, *Sirex cyaneus*, is in the wood of coniferous trees. In each case only the ovipositor can penetrate to the host. This would suggest the operation of the second faculty, connected with the ovipositor, but Chrystal (1930 p. 42) has observed that "*Ibalia*, once she has used an oviposition tunnel, frequently crosses and recrosses it seeking for other tunnels in which to oviposit." He goes on to suggest "It is probable, therefore, that she recognizes those tunnels which she has already visited, and possibly she leaves some odour behind her by which other females are also furnished with this information." This suggestion is given weight by the results now obtained with *Trichogramma*. Henceforth, when a parasite of burrowing hosts is found to avoid superparasitism, the occurrence of a chemical trace on the surface of the plant is at once to be suspected.

In a recent paper (Salt 1936) it has been shown that, even when hosts are very scarce and superparasitism is rife, one or two hosts sometimes escape parasitization by *Trichogramma* and emerge as larvae. This escape of a few hosts, when there are theoretically more than enough parasites

present to annihilate them, is very important both to the student of animal populations and to the economic entomologist. The present study shows how the escape may come about. If a host is examined by a parasite which then, either because it is disturbed or because it rejects the host, moves away without parasitizing it, that host is protected by its odour from further attack. It was probably in this way, and not because they were not found by the parasites, that a few hosts escaped in the experiments of the paper cited above. Perhaps hosts of other species escape parasitization in nature by a similar mechanism.

Finally, the result of this study serves to reconcile two groups of facts previously reported. The conclusion of the second paper in this series that ovipositing females of *Trichogramma* are able to distinguish healthy hosts from those already parasitized seemed to indicate that this parasite has a highly developed faculty enabling it to select hosts suitable for its offspring. The observation of the third paper, however, that these same parasites accept and attempt to parasitize many kinds of false hosts quite unsuitable for their progeny provided only that those objects are of the right size seemed to contradict that supposition and to suggest that the parasite has little or no ability to distinguish suitable from unsuitable hosts. The present investigation shows that there is no inherent contradiction in those conclusions. The parasite accepts and tries to parasitize false hosts if they comply with its conception of a host. Similarly, it rejects and fails to parasitize true and unparasitized hosts if they bear the inhibitory odour left on them by a previous parasite. In short, *Trichogramma* selects its hosts by a series of criteria which are not necessarily criteria of suitability. There is no need so far as the analysis of this problem has yet gone, to postulate a mysterious instinct perceptive of host suitability.

An incubator and accessories furnished by the Government Grant Committee of the Royal Society continue to be indispensable for this work. The later stages have been much aided by a grant from the Department of Scientific and Industrial Research which provides the assistance of Mr E. B. Basden. Now, as before, my thanks are due to Dr James Gray, F.R.S., for encouragement and help.

#### SUMMARY

It has already been shown that ovipositing females of *Trichogramma evanescens* are able to distinguish healthy hosts from those already parasitized. The present study deals with the sense used in the discrimination

Sight hearing and touch are eliminated and the chemical sense is indicated as the one used

The parasite is able to distinguish between clean hosts and hosts that have been merely walked upon not stung or oviposited in by another female of its species. If hosts that have been walked upon are washed in water the parasite can no longer distinguish them. The means of discrimination is of a chemical nature and being volatile can be considered an odour

If hosts that have been actually parasitized are washed the parasite is unable to distinguish them externally from healthy hosts and attacks them. As soon as its ovipositor has penetrated into them however the parasite becomes aware that they are parasitized and usually withdraws immediately without laying an egg in them

Two different faculties then can perform the discrimination. One recognizes an external odour and inhibits attack the other distinguishes an internal difference and inhibits oviposition

The external odour passes off in time but the internal means of discrimination is lasting

The chemical trace left on the surface of the host is not the general body odour of the parasite but a more specific smell probably produced by glands on the tarsi. It is left not only on hosts but also on the substratum on which the parasite walks

Owing to its method of discrimination the parasite sometimes mistakes healthy for parasitized hosts. These therefore escape parasitization and are important in the population problem

The paper affords a further demonstration that *Trichogramma* selects its hosts by a series of criteria which are not necessarily criteria of suitability

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# The Structure of the Wall of the Green Alga *Valonia ventricosa*

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[Plates 1-2]

## INTRODUCTION

Although for many years the study of cytology has tended to concentrate attention more and more on the protoplast as the fundamental unit of the plant, there can be no doubt that the membrane surrounding this unit plays a part of considerable importance in its life processes. The deposition of such a membrane by a process which is as yet quite obscure is obviously closely connected with protoplasmic activity, and a detailed investigation of its structure is bound to lead to a better understanding of this connexion. At the same time the shape and size of a cell are clearly due in some degree to the action of forces external and internal on the membrane, so that a study of the structure of the plant cell wall should therefore also yield information of considerable importance in the solution of botanical problems concerned with cell elongation and growth. Comparatively recent investigations carried out chiefly on plant fibres have shown that the most important component of cell walls from a structural point of view is the polysaccharide cellulose. This substance is known to occur in varying proportions in the walls of almost all plant tissue and its structure has been worked out chiefly by X-ray and chemical methods, with some degree of certainty. Although much remains to be discovered of the organization of cellulose in the wall, certain details are now quite clear. Celluloses obtained from many and varied plant sources have all proved to have essentially the same structure. They exist only in the form of chains of  $\beta$  glucose residues at least 500 Å long (Hengstenberg and Mark 1928), bound together laterally by secondary valences to form a three-dimensional lattice. The conception of a definite micelle in the sense of Nägeli is no longer widely held, although the lattice is not uniformly regular throughout the wall. The chains of cellulose are more probably bound together into ill-defined bundles separated by regions in which they are not so perfectly oriented.

This conception of the existence of cellulose in long molecular chains has arisen from the examination of the secondary walls of plants, but as yet no direct experimental determinations have been possible of its structure in primary walls where it is known to occur (e g in *Vicia faba*, see Tupper Carey and Priestley 1922). Recent work (Preston 1934) on the tracheids of the conifer however, show that it is possible to carry over the idea of the long chain structure of cellulose even to these delicate primary walls.

This widespread distribution of cellulose with essentially the same structure makes it possible to generalize results obtained on the wall of one type of cell to cover that of many other types and it is from this point of view that the work described below will be of interest to botanists. It is possible to make observations on the large cells of *Valonia* which imperfections of technique make impossible with the minute cells of the higher plant. Moreover accurate observation of the structure of the whole wall which can be made only on large cells such as this alga affords will probably yield results with an important bearing on the problems involved in the deposition of the cellulose wall at the protoplasmic surface.

*Valonia* is a member of the Siphonales (Fritsch 1935) characterized by its bubble like cells, which in some species may be two or three centimetres long and which are found in the warmer seas sometimes in apparently irregular clusters and sometimes in the form of neat palisades. Of the three species used in this investigation *V ventricosa* and *V macrophysa* form usually spherical or pear shaped cells which in the former generally occur singly, being larger than the proliferating cells of *V macrophysa* while *V utricularis* proliferates freely giving close clusters of cells which are relatively smaller and frequently somewhat elongated. The bulk of the work to be described below has been carried out on *V ventricosa*, although sufficient observations have been made on the other two species to show that their cell wall structure is essentially similar.

A brief account of the morphology of the alga *Valonia* has been given in the first paper of this series (Astbury, Marwick and Bernal 1932), but it becomes necessary here to enlarge upon this outline. The following summary is based on Oltmanns (1922) and Fritsch (1935), to whom reference may be made for further details. The cell may be imagined as a large bubble, often approximately spherical with a large vacuole and a thin lining of protoplasm. Imbedded in this lining are to be found numerous nuclei, while further away against the cell wall occur the chromatophores, in the form of plates of irregular outline often united to form a network and frequently containing pyrenoids. The protoplasmic lining is in turn completely surrounded by a comparatively thick wall, consisting chiefly of cellulose.

*Valonia* is a coenocytic organism with the protoplast contained in large vesicular cells, but minute cells are often formed as a result of accumulations of protoplasm in certain regions of the surface. A strongly curved wall, shaped like a watch glass, is formed round such protoplasmic masses giving a cell with a characteristic appearance. This process gives two kinds of cells, larger ones which appear in the upper part of the cell, and smaller ones which occur particularly at the base. The latter grow out into short, lobed, but single celled structures which form the holdfasts, while the larger cells on the upper part grow out into new bubble like structures which resemble the parent in every way, including the power of forming new cells.

The reproduction of cells by the formation of zoospores has been closely observed and described by several investigators. The propagation of zoospores is made obvious several days before their ejection by the various localized changes occurring in the wall and protoplasm of the mother cell (Kuokuck 1902). The fertile plasma is not separated from the rest of the plasma by a wall as it is, for example, in *Vaucheria*, *Bryopsis*, etc., but the vacuole is in direct communication with the outside environment at the time of spore ejection. Previous to ejection, the wall is completely pierced, the opening being subsequently closed up and the mother cell regaining its original condition. There seems to be no evidence that this spore formation takes place under any particular area of wall. Certainly in the present research no disturbances in wall structure have been found such as one would expect *a priori* from such openings in the membrane. Either the cell under investigation had never produced zoospores or the perforations are closed up in such a way as to leave no trace of their existence.

In the course of the work to be described below it has become clear that the wall structure of the cells of *Valonia* is strikingly similar to that of the fibres of the higher plant. The wall is laid down in microscopically visible layers, which may be as many as thirty or forty in number, and the crossed cellulose chains previously described are found, as a result of taking numerous X ray photographs of the same cell, to be portions of two complete sets of chains traversing the whole wall surface. Of these two sets one forms a left hand spiral round the cell, while the other takes the form of meridians running from one pole of the spiral to the other. At the two poles of the spiral, therefore, the typical X-ray photograph of *Valonia* is no longer obtained, being replaced by a Debye Scherrer ring diagram. Moreover, these two sets of chains correspond to the microscopically visible striations in the wall and occur in separate layers rather than in the same layer. The existence of the striations in different layers had been already

indicated in the work of Correns (1892) and it has been verified during the present research. The view put forward by Sponsler (1931) that the chains are definitely oriented about their axis, with the planes of 61 Å spacing always parallel to the wall, has been shown to be only roughly true. The chains do tend to lie in this position, but there is a considerable dispersion.

Apart from the disturbances due to the poles of the spiral, the only breaks in the regularity of the wall structure occur at well defined places where "watch glass" cells have been formed. At the holdfasts and at the scar left by the falling off of a bud cell the wall undergoes some modification. The region of the holdfasts show a series of raised circular rims some  $\frac{1}{16}$  mm diameter which present a crater like appearance under the microscope when illuminated parallel to the wall surface. The two sets of striations on the wall inside each rim are continuous with those outside it, although a piece of wall containing a rim gives a Debye-Scherrer ring diagram in the X ray microcamera (see Preston 1934). Bud scars have a similar appearance, though on a much larger scale.

#### STRIATIONS AND WALL LAYERS

Although in the first paper of this series a conclusive demonstration was given of the correspondence between the directions of the striations and those of the cellulose chains, it may not be out of place here to enlarge upon this point. The results presented in this connexion will serve in particular to indicate the order of precision in the various interrelations between the cellulose chains, extinction positions, and striations.

Observations have been made on the striations on many pieces of *Valonia* wall taken from several cells and in only one case was there observed an obvious discontinuity in direction unaccounted for by a fold in the wall (special reference to this exceptional specimen will be made later). The constancy in direction of the striations can readily be observed under the microscope in any piece of wall, although each striation is not equally visible at all points, and this necessarily implies that the angle between the striations must also be constant. While separate specimens have been obtained with interstriation angles varying from 60° to 80° or more, the angle in any one specimen varies in a strikingly gradual fashion. This will be clear from Table I, which gives a series of readings on a single small piece of *Valonia* wall. The results of many measurements, of which Table I is a representative sample, indicate that in general the striations travel in lines which are to a close approximation straight over a distance of several

millimetres In particular, the area covered by the slit of the X-ray spectrometer (diameter 0.5 mm.) is uniform in this respect.

TABLE I

mm intervals	← 1 mm intervals →				
	1	2	3	4	5
<i>a</i>	83.6°	84.4°	76.0°	84.2°	76.4°
<i>b</i>	81.6°	86.6°	76.4°	85.0°	74.6°
<i>c</i>	83.6°	86.0°	83.0°	83.6°	85.8°
<i>d</i>	81.6°	85.2°	83.8°	85.4°	84.8°
<i>e</i>	87.0°	84.2°	78.4°	86.2°	84.0°

This remarkably uniform nature of the striations alone would indicate that they are reflections of inner structural details of the wall, but a series of observations was carried out on the X-ray spectrometer and under the microscope in order to demonstrate the correlation still more completely. A method was used similar to the demonstration given in the previous paper. Small areas, about the size covered by the spectrometer slit, were marked out on a piece of wall and the directions of the cellulose chains were determined by the X-ray method. These were plotted on paper together with the corresponding striations and, wherever possible, the major extinction position. In some cases the region examined consisted of "mosaic" areas (Preston 1931) too small to allow the determination of a representative extinction position, and in others one of the two sets of striations was too indistinct for exact determination of its direction. In spite of this, a sufficiently large number of observations was made to show conclusively that the sets of striations are parallel to the cellulose chains, and that the major extinction position lies in the acute angle between them. It is true that in some cases there is a discrepancy of a few degrees, but we cannot expect exact agreement every time owing to the frequent indistinctness of one set of striations. Bearing this point in mind, the correspondence between the directions of cellulose chains and striations is found to be extremely close. Typical results are presented in figs 1 *a*, *b*, from which several further conclusions may be drawn. In every case, the more easily visible set of striations corresponds to the set of cellulose chains giving the more intense diffraction spots. The striations not only indicate the directions of the two sets of cellulose chains, but they afford also a qualitative measure of their relative importance. Again, the figure shows conclusively that the major extinction position lies in the acute angle between the cellulose chains, and always closer to the more important set. This, of course, is what we should expect from the multi-ply structure

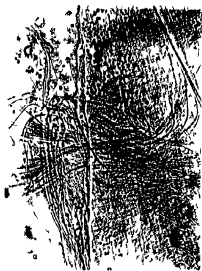


FIG 2

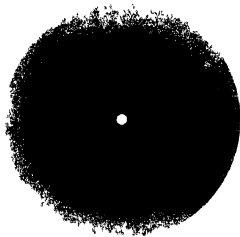


FIG 6



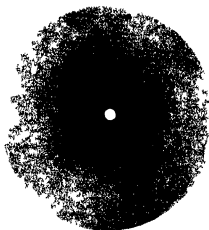
FIG 5



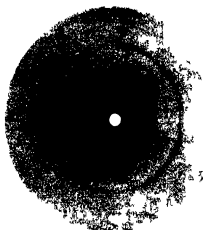
Pl 7



Pl 8



Pl 9



Pl 10

described below, a structure which is in effect a series of superposed birefringent plates with extinction positions not coincident. Attention may be drawn specially to fig. 1*b*, representing a set of observations on neighbouring areas of a piece of the wall. In area *A* the major extinction position lies about  $15^\circ$  to the left of the more important set of chains, while in *C*,

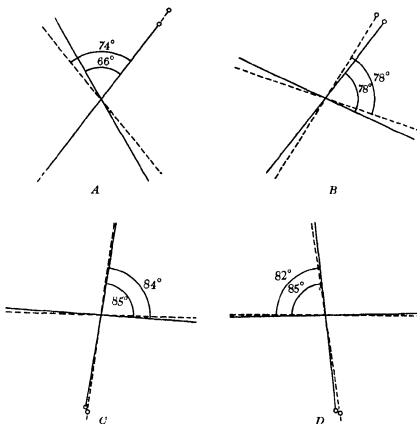


FIG. 1*a*—Directions of cellulose chains and striations on arbitrary pieces of *Valonia* wall

3 mm away, it lies some  $30^\circ$  to the right. *B*, on the other hand, represents the only observed specimen in which an abrupt change occurred in the direction of the striations. At this point there existed a definite boundary between two areas, each with its own striations. From *A* to the "frontier" the striations behaved normally, but in this region they changed over abruptly to those in *C*, and the extinction position altered simultaneously in



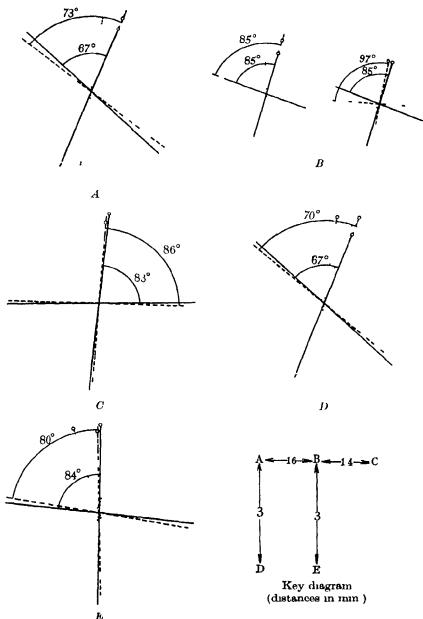


FIG 16—Directions of cellulose chains, striations, and major extinction positions at various points on a single piece of *Valonia* wall (see key diagram). At B, the frontier region (see text) the two sets of striations and major extinction positions are drawn separately for clearness. At D and E the limits of variation of the major extinction position are as indicated. Cellulose chains ——— striations - - - - - , more important sets —○— - - -○- - - ○

*a corresponding manner* The change in the direction of the striations corresponds to a similar change in that of the cellulose chains and there can be no doubt that the direction of the extinction position at any point is determined partially by the *direction* of the chains

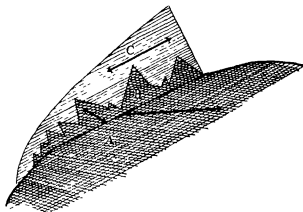
Areas *D* and *E* are no less interesting from another point of view. Although each piece consisted of mosaic areas much smaller than the area included in the X ray beam and the major extinction position varied over a considerable angle from one mosaic area to the next only the usual two sets of cellulose chains and striations could be detected. It is therefore obvious that the direction of the major extinction position is determined not only by the directions of the cellulose chains but also by the proportions of the two sets present in the wall thickness. One small area in *E* showed a major extinction position exactly parallel to the more obvious set of striations (which was unusually pronounced compared with the second set in the area) this particular area therefore was structurally different from the rest of the wall in that one set of chains was almost entirely absent. The majority of the mosaic areas however undoubtedly arise from varying proportions of two sets of chains in the wall thickness and this single case represents one of the limits of variation. Perhaps a point raised in the previous paper may again be emphasized. Any work on biological structures carried out under the polarizing microscope alone must be regarded with suspicion until confirmatory evidence has been obtained such as is afforded by the X ray method.

We have therefore a wall consisting of many microscopically visible layers and corresponding to a network of cellulose chains making an angle of some  $80^\circ$  with each other. Complete understanding of such a structure is obviously impossible without an investigation of that of the individual layers—to decide whether even the finest layer has a structure similar to that of the whole wall or whether the wall is composed simply of more or less alternating layers each with only one direction of cellulose chains. The work of Correns (1892 quoted from van Iterson 1933) supports the latter alternative. Correns concluded from careful microscopical examination that the odd layers had one set of striations while the even layers had the other. If this is true then any one layer cannot have everywhere the same thickness. The directions of the extinction positions vary from point to point a change which is necessarily connected with the relative amounts of the two sets of chains. If then the same *number* of layers of each kind are present in two neighbouring mosaic areas their relative thickness must vary.

This work of Correns has now been verified by physical means. As yet

no direct tests of the structure of a single lamella have been possible. Extremely thin lamellae can be stripped from fresh cells, but even these fail to show any indication of a structure different from that of the whole wall. It has not yet been found possible to strip off a layer with a single set of chains. On the other hand, indirect evidence does certainly support the work of Correns.

As shown by van Iterson, Jr (1933), pieces of *Valonia* wall can be torn in such a way that the torn edge exhibits a fringe of fibrils. Here and there, the otherwise straight edge of the wall is interrupted by sets of these fibrils pulled out from the wall. van Iterson gives a drawing showing a small



← —→ Major extinction position

Fig. 3

piece of wall standing out from a torn edge and with such a fringe of fibrils. This small piece shows only a single set of striations, which in the drawing are obviously the origin of the fibrils, and is therefore a single layer in the present sense. It has been found impossible to repeat this observation exactly. Many pieces of wall have been subjected to a treatment similar to that of van Iterson, but in no case was it found that the fibrils at the torn edge originated from the set of striations *perpendicular* to the edge. On entering the wall the fibrils obviously turned through a considerable angle, and finally were lost among the striations parallel to the torn edge. Fig. 2, Plate 1, makes this clear. It would seem that the fibrils perpendicular to the torn edge are the first to break, leaving the two pieces joined together by the lateral fibrils which are then pulled out before breaking.

In fig. 3 is given a diagrammatic representation of a second type of observation that may be made at a torn edge. Such an edge often shows

a terraced effect due to the stripping off of various numbers of wall layers. The portion of wall illustrated is a particularly interesting example of such a phenomenon. Three distinct regions can be seen: *A* which represents the whole wall thickness; *B* where only a few layers are left; and *C* which is probably a single layer. It is unfortunately impossible to present an actual photograph of this specimen since attempts completely to flatten the wall for distinct focusing in the camera caused this part to break up into fibrils. The striations and extinction positions marked in the figure however make it quite clear that the removal of several wall layers has caused a change in the orientation of the major extinction position and that in the region *C* the layer consists of a single set of cellulose chains. There can be no doubt that the layers are not identical with one another and we may fairly conclude that their structure is distinct from that of the whole wall in that each is built from one set of cellulose chains. The whole wall is composed of a series of superimposed layers each with its own cellulose chain direction.

Thus it would seem that in *Valonia* both striations and layering are definitely related to structural details in the wall. Now although the external form of the *Valonia* cell is widely different from that of the fibres of the higher plant the structure of its wall is essentially the same. This will be clearly demonstrated below. The present results therefore give further support to the generally accepted view that in general whenever striations are visible on the walls of cells of the higher plant (e.g. phloem fibres, xylem fibres and tracheids, cotton hairs) they are not merely artefacts but correspond closely to the structure of the walls. This is undoubtedly true for the walls of certain conifer tracheids since Frey Wyssling (1930) has observed striations parallel to the major extinction position which in turn have been shown to be parallel to cellulose chains (Preston 1934).

The exact significance of the striations and layering of the plant cell wall has been the centre of considerable discussion for many years. Many cases of cell walls with crossed striations have been quoted notably by Reimers (from Steinbrinck 1927 and Herzog and Jancke 1928). These observations refer almost exclusively to phloem fibres (e.g. of hemp, hop, ramie, flax) in which the wall layer showing one of the sets of striations usually predominates. The conception of Nageli that the appearance of striations is caused by regions of high and low water content has been rejected by Dippel (1879), Schmitz (1880), Strasburger (1898) and Krabbe (1887) who agreed that in phloem fibres the striations are merely distorted contact faces between adjacent screw bands in intimate contact. These authors also contested Nageli's observation that two sets of striations can appear in one layer of the wall. Their view of the origin of striations has in turn been

rejected by Correns (1893) as physically impossible he is of the same opinion as Nägeli. Wiesner (1892) again put forward a third hypothesis in which the wall is composed of Dermatosomes which aggregate to form both fibrils leading to striations and layers. He considered these Dermatosomes to be separated by layers of some protein or its derivative a residue of the original protoplast of the cell but repeated experiments by Correns have failed to show any trace of protein in the wall. The primary cell walls of plants certainly contain a protein complex (Tupper Carey and Priestley 1923) but there seems to be no evidence for any considerable amount of protein in the secondary layers such as are under consideration here.

There can be little doubt that the effect of difference in water content on the visibility of layers and striations is only of secondary importance and is inseparably connected with a difference in chemical constitution. Hess, Ludtke and others (van Iterson 1933) have been led on the basis of swelling experiments to the assumption of partitions of non-cellulosic substances between the wall layers and even the fibrils and the fact that this conception fails to account for certain phenomena does not invalidate their argument outright. It is interesting in this respect to note that Farr and Eckerson (1934) have recently observed in the protoplasm minute bodies which they describe as cellulose particles surrounded by a layer of pectin although the significance of this observation is perhaps open to question (Bailey and Kerr 1935). At the same time the view that striations are due merely to the separation of fibrils by less perfectly oriented regions of the same composition cannot be entirely disregarded.

#### THE ORGANIZATION OF THE WALL AS A WHOLE

None of the observations presented above suggests any fundamental difference between the cell wall of *Valonia* and that of the fibres of the higher plant in spite of the difference in cell size and the correspondence is again evident when we come to consider the details of the organization of the wall as a whole.

The modification of wall structure which must occur at the tips of cells whose walls are wound with a molecular spiral has hitherto been a point of mere conjecture and any investigation throwing light on this subject cannot fail to be of considerable value. The opportunity was taken therefore of carrying out a survey of the whole *Valonia* wall. The uncertain visibility of the wall striations made it impossible to follow microscopically

their directions uninterruptedly round the cell so the investigation had to be carried out by X rays

A herbarium specimen of *V. ventricosa* collected at St Croix and sent to us by Borgeson to whom our thanks are due was emptied of its contents through a small perforation. Into this perforation a fine glass capillary tube was inserted and fastened in place by a minute ring of cellulose cement whence by alternate emptying and filling of the cell with distilled water the remains of the protoplast etc. were finally ejected. Incrustations clinging to the outside of the wall were removed by subsequent treatment with N/20 HCl. When dry the cell was sufficiently rigid to be mounted on the X ray spectrometer by clamping the capillary tube to a brass arm with a universal joint. By careful adjustment any part of the wall could thus be set perpendicular to the X ray beam. In order to obtain reference lines whereby the directions of the cellulose chains as given by the X ray photograph could be transferred to the cell itself the following procedure was adopted. The cell was mounted on a spindle by means of which it could be rotated and raised through measured distances and a series of lines some 3 mm. apart and forming complete circles round the cell was traced in Indian ink using a modification of the usual inking system of barographs etc. A pair of straight wires was then attached to the spectrometer so that they could be set parallel to that part of a line on the cell nearest to the area under examination and would cast a shadow on the photographic film. In general this area under examination was arranged just to touch the spectrometer slit while the photographic film was placed as near to the other side of the cell as possible. It was then quite a simple matter to differentiate between the diffraction spots produced by the two opposite sides of the cell (see fig. 4).

In order to obtain a map of the whole wall a method was used similar to the familiar lines of force method of mapping magnetic fields. The chain directions were determined at an arbitrary point in the wall and were then drawn upon the wall. Now previous experience had shown that the direction of either set of chains was almost constant over a length of  $1\frac{1}{2}$  mm. A second point was therefore chosen  $1\frac{1}{2}$  mm. from the first in the direction of one of the sets of chains and the directions again determined. This process was continued round the cell using only one set of cellulose chains. In general no difficulty was experienced in determining which of the two directions at a new point corresponded to the one being traced any uncertainty where it arose being entirely removed by the investigation of intermediate points.

A starting point was chosen about midway between the base and tip

of the cell, where one set of chains was found to lie approximately along a line joining the tip and the base. On following this direction, the chains were found to form a great circle round the cell, passing amongst the holdfast scars and across the cell apex. Unfortunately, the value of this set of observations was somewhat reduced by very considerable dispersion of the X ray diffraction spots in certain regions, particularly near the holdfast scars and the cell apex. Investigation of the second set of chains, however, confirmed this result in a very striking manner. At each point on this

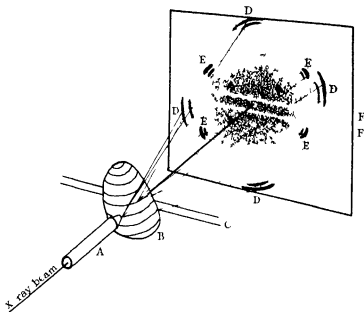


FIG. 4.—Illustrating the method of transferring cellulose chain directions from the photographic film to the cell itself. *A*, spectrometer slit; *B*, *Valonia* cell; *C*, two parallel wires; *D* and *E*, diffraction spots produced by the part of the wall nearer to and further from the slit, respectively; *F*, shadows cast by the wires; *C'*.

second track both directions of cellulose chains were marked in ink upon the wall, although only one of them was followed. The second chain direction was thus found to make a slow spiral round the cell, the turns of the spiral becoming smaller as the apex and base of the cell were approached until finally both at the apex and the base a point was reached where the X ray photograph characteristic of *Valonia* was no longer obtained. Both points were strictly localized and gave a photograph consisting of a series of rings such as is obtained from a crystalline powder. It is important to note that these two "poles", as they may be termed, were discovered not by accident

or by a method of trial and error, but by painstakingly following the spiral set of chains round the wall. They would appear to be produced by the wall deposition mechanism of the plant rather than by any local, accidental change in environmental conditions. A model of the structure of the *Valonia* wall is shown in fig. 5, Plate 1, in which one "pole" can be seen towards the upper end. The X-ray photograph of this "pole" is reproduced in fig. 6 Plate 1. It may be pointed out that the second set of chains recorded at each point of the spiral may be linked up with chains immediately above and below and that the circles thus obtained form, so to speak, "meridians" uniting the two "poles". Whether it is an invariable rule that one set of chains always forms great circles uniting the tip and base of the cell, as in the present case, is not yet clear. A decision on this point is best made upon a long, narrow, cylindrical cell and only one of this type was available. In this one specimen, however, one set of chains was observed to run approximately along a "meridian" at all points of the wall which were investigated. We may thus picture the *Valonia* cell wall as consisting of two crossed sets of cellulose chains, one running in great circles (possibly always from base to tip and back), and the other forming a slow spiral round the cell axis joining the two points of intersection of these great circles.

It has been pointed out above that at the base of the cell, near one of the "poles", there occurs some disturbance in the otherwise regular appearance of the wall surface. In this region clusters of raised, rim like structures may be observed which obviously mark the sites of previous rhizoids (holdfasts). In some few cases in the specimens available the rhizoids can still be seen attached to the cell in the form of long narrow, and hollow cylinders widening into trumpet shaped attachments at the point of connexion. Such a rhizoid may be seen in the photomicrograph of the surface of the basal region of a cell shown in fig. 7, Plate 2. The preparation shown in this figure was stained in methylene blue to bring out the fact, which is perhaps more obvious in cross section, that the wall is much thinner inside the rims than outside. This no doubt explains the X ray photograph obtained in these rhizoids. If we choose a rim of such a diameter as just to be included in the X ray beam then the X ray diffraction pattern obtained appears to arise solely from the rim. Although the wall inside the rim seems to be identical in structure with that outside, and the striations on it are continuous with those on the rest of the wall, its thickness is so small compared with that of the rim itself that its X ray photograph does not mask that of the rim. The X ray microphotograph of such a rim was found to consist of a series of concentric rings, indicating



random arrangement of the cellulose particles. This is exactly what we should expect from consideration of cross sections of the wall. Fig. 8, Plate 2, is a photomicrograph of such a cross section. Here the remains of a rhizoid are seen clinging to the wall (on the left) and located immediately above a small cell cut off from the parent. The whole structure is filled with small granules which appear to be plastids surrounded by a comparatively thick layer of starch, and may perhaps play a part in the development of the rhizoid. It is clear from the photograph that the raised rim seen in surface view consists merely of a ring of the outer layers of the wall turned on edge, and the X ray photograph is in effect that of a cross-section of a cylindrical holdfast.

The structure of the rhizoids as illustrated in figs. 7 and 8, Plate 2, and by the X ray microphotograph, is in complete agreement with the descriptions given by other workers (Famintzin 1860, Borgesen 1905). It is quite clear that the cylindrical outgrowths originate as small cells cut off on the inside of the "main" vesicle by surrounding a small collection of the necessary plasma masses by a strongly curved subsidiary wall, before the deposition of the wall of the mother cell is complete. As more and more layers are deposited over this "watch glass" wall, by the continuous deposition of new wall substance by the parent cell, it becomes eventually buried in the wall. It may well be that the wall on the outside of this small cell is then considerably thinner than that on the inner side. At the same time it must be noted that the inner wall borders, not on the open sea but on a virtually incompressible interior supported by comparatively firm walls. It is not surprising therefore, that if the "watch glass" cell begins to expand the expansion takes place towards the outside. This would explain the formation of both rhizoids and bud cells on the outside of the parent plant. Here however we meet with a difficulty. Whereas the bud cells are usually almost spherical in form, rhizoids are always produced as long narrow cylinders. Hitherto no explanation of this divergent behaviour of essentially similar cells has been possible. Now that the wall structure of the plant has been determined, we find that the rhizoids arise from regions of the wall adjacent to the poles of the spiral, and it is not unreasonable to suppose that the difference in behaviour of "watch glass" cells is connected with the difference in wall structure. Such a connexion could, of course, be traced only in the vaguest terms at present, and its investigation is a subject for further research.

## RELATION OF CELLULOSE CHAINS TO WALL SURFACE

It has been mentioned already that the conclusion arrived at by Sponsler (1931) that in *Valonia* the planes of 6.1 Å spacing lie parallel to the wall surface is only partly justified. In the course of the present research it became clear that any X ray results obtained from blocks built up by superposing many pieces of the wall are liable to be misleading. It was thought advisable therefore to reinvestigate the question of the angular dispersion of the cellulose chains using single pieces only. To this end a small area of wall was selected on which one set of striations predominated and whose X ray photograph showed a preponderance of one set of chains over the other. Fig. 9, Plate 2, is an X ray photograph of the area chosen. One set of reflexions is so much more intense than the other that for the purpose of studying its angular dispersion the weaker set may be disregarded.

The most direct method of demonstrating the angular dispersion of the cellulose chains is illustrated by the photograph shown in fig. 10, Plate 2, for which the flat piece of wall was mounted horizontally on the spectrometer with the main set of chains parallel to the X ray beam. If now as Sponsler suggested the cellulose chains had been lying in only one orientation round their axis, not arcs but spots as definite as those in fig. 9, would have appeared in the photograph. The photograph reveals in fact quite a considerable dispersion for both of the inner sets of arcs (corresponding to planes of spacing 6.1 and 5.4 Å) can be traced round a complete circle. The intensity however certainly does decrease rapidly at fairly definite limits and roughly speaking it may be said that the normal to the plane of spacing 6.1 Å is confined to about 60° on either side of the normal to the wall surface.

## DISCUSSION

The remarkably regular organization of the wall of such a large cell as that of *Valonia* is perhaps the most interesting result of this research. The fact that the cell has a structure fundamentally similar to that of the minute fibres of the higher plant serves once more to emphasize the essential unity underlying biological phenomena. It has long been a question whether coenocytic cells such as we have under examination here could be regarded as single units comparable with the protoplasts of higher plants containing but a single nucleus but as regards the wall at least there can no longer be any doubt about this. The wall of *Valonia* would appear to be just as

uniform in structure as that of uninucleate cells and can be regarded only as that of a single cell

The appearance of crossed striations is of course not a novel phenomenon in wall structure. It is widely recognized that in bast fibres the secondary wall is laid down in definite layers and that these layers can be striated in different directions. For example fibres from hemp and hop plants have two secondary layers the striations on both running round the cell in a right hand spiral with the spiral on the outer layer less steep than that on the inner. On the other hand bast fibres from flax and oleander show definitely crossed striations the spirals on the two layers being of opposite sign. Moreover in both types of cell the view put forward by Nageli that crossed striations can appear in a single wall layer is no longer held. As has been shown above in the case of *Valonia* striations in different directions invariably occur in different wall layers. Here however the similarity between *Valonia* and fibres effectively ceases. Whereas in bast fibres change in spiral sign occurs but two or three times and the structure of one layer is not repeated in a subsequent layer *Valonia* has numerous layers which alternate regularly in striation direction in a very exact manner. This deposition of alternate layers each with the same direction of molecular chains as the last layer but one presents perhaps the most intricate problem in wall formation as yet encountered in botany. It seems impossible without serious modification to invoke the idea of pseudo-crystallization of new substance on an old wall such as is often put forward in discussions of wall deposition at the least it must be recognized that the growth mechanism involves a periodic halt in the effectiveness of an old wall in orienting new layers.

The existence of mosaic areas is to be explained on the lines already laid down. They arise as a result of variations from point to point in the proportions of the two sets of cellulose chains in the wall thickness. It seems reasonable to suppose that these variations are due rather to a varying thickness of the wall layers than to a fluctuation in their number. The mechanism underlying the formation of mosaic areas is no doubt to be sought in fracture of the wall during development as already suggested by one of the present writers (Preston 1931) \*

With regard to the geometrical form of the path followed by the spiral set of cellulose chains it would appear that this approximates most to an

\* This idea is supported by the fact that remnants of wall layers presumably the original outer layers are often found clinging to the plant when collected. This has been pointed out to us by Dr Steward of Birkbeck College London who recently had the opportunity of studying *Valonia* in its native habitat.

equiangular spiral described on the surface of a spheroid Fig 11 illustrates a prolate spheroid, which is a reasonably fair description of many *Valonia* cells. If the spiral at any point  $(\theta, \phi)$  of its path makes a constant angle  $\alpha$  with the meridian  $(\theta = \text{constant})$  through that point then

$$\cot \alpha = \delta s / y \delta \theta$$

or

$$\theta \cot \alpha = \int ds / y$$

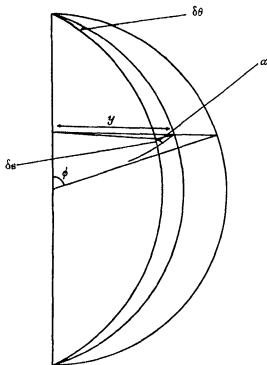


FIG 11

the solution of which if  $\theta = 0$  when  $\phi = \pi/2$  is

$$\theta \cot \alpha = -\frac{\sqrt{a^2 - b^2}}{2b} \cos^{-1} \left[ \frac{b^2}{a^2} - \frac{a^2 - b^2}{a^2} \cos 2\phi \right] - \frac{1}{2} \cosh^{-1} \left[ \frac{2b^2}{a^2} \cot^2 \phi + 1 \right]$$

For the sphere this reduces to

$$\theta \cot \alpha = \log \tan \phi / 2,$$

as may be readily derived directly

This general formula is given here in case experimental opportunity should arise later of testing it rigidly

It should be noticed that  $d\phi/d\theta = 0$  when  $\phi = 0$  and  $\pi$  and is a maximum when  $\phi = \pi/2$  as was actually observed for the *Valonia* cell described above

Owing to irregularities of growth it is hardly a practical proposition however to make a strict quantitative test of the equation though it may be that careful examination of more abundant material than was available for these experiments would reveal specimens sufficiently perfect At the moment we are justified in saying only that the angle  $\alpha$  is roughly constant and not far removed from a right angle—the mean of the values given in Table I for instance is about  $83^\circ$  and that the spiral it produces the main features of the path of a point moving on the surface of a spheroid so as to make a constant angle with the meridians

The approximate constancy of angle between meridians and spiral that is maintained through alternate layers and the fact of alternating deposition itself seems best explained for the time being in terms of a rhythmic orienting mechanism embodied in the polynuclear protoplasmic lining Recent observations on the cytoplasm of algae those of Chadeaud (1933) for instance are strongly suggestive of such a mechanism and the following extract from Chadeaud's paper 'Existence d'une structure infra visible orientée du cytoplasme chez les Algues' is very much to the point

L'existence d'une structure orientée du cytoplasme se traduit d'une façon encore plus intéressante dans les grandes cellules allongées du tissu central de *Chorda filum* Le cytoplasme de ces cellules possède deux séries de lignes directrices à peu près orthogonales et fortement inclinées par rapport à l'axe longitudinal de la cellule l'une de ces directions est prépondérante elle oriente la plus grande partie des phéoplastes des chondriosomes et des amas de physodes et tous les noyaux qui sont étirés en fuseau Quelques phéoplastes seulement sont orientés selon l'autre direction Or il est très curieux de remarquer que ces deux directions de la structure cytoplasmique coïncident avec celles des deux systèmes de fines stries que présente la membrane celluloso-pectique et que révèlent de façon très nette les ponctuations en X de cette membrane On trouve ainsi une relation évidente entre la structure cytoplasmique et celle de la membrane cellulaire

The occasional appearance of a third orientation lying between the two predominating sets of cellulose chains is possibly a manifestation of attempts to set up a spiral of opposite sign and may represent still another link with the structure of the fibres in which the occurrence of spiral reversals is fairly common

van Iterson (1936) considers that the approximate orthogonal relation between the cellulose crystallites in adjacent layers of the wall of *Valonia*

is simply a consequence of an alternation of wall stretching and protoplasmic streaming, the direction of easiest stretch being at right angles to the length of the crystallites already laid down. The periodic stretching would be caused by the strong increase each day in the turgor pressure in the cell for example, and it is supposed that the stresses so set up determine the direction of flow of the protoplasm as it deposits the next layer with the crystallites lying along this direction of flow. The concept perhaps marks an advance in the sense that it offers something rather more concrete to work on, but it still leaves vague the initiation of the process and does not explain how stretching can take place in a multiply structure first in one direction and then in a direction at right angles. The idea may be valid for a wall consisting of two layers only but it is not easy to see how the mechanism continues to operate with such angular regularity beyond this stage. In any case, the impression gained from the studies reported above is that the average angle of crossing is definitely less than a right angle, though to be sure it might be possible to trace this deviation to some secondary source.

Finally, reviving once more to the question of the approximate orientation parallel to the cell wall of the planes of spacing 61 Å a recent paper by Sisson (1936) is very illuminating. From an X-ray study of the various types of crystallite orientation that can be brought about artificially in membranes of bacterial cellulose Sisson concludes that in general whenever a sample is constricted in one direction, whether by drying or by pressure, then the cellulose crystallites have an inherent tendency to set themselves with the planes of spacing 61 Å normal to the direction of constriction. It would appear, therefore, that to explain in *Valonia*—or in any cellulose wall for that matter—the observed selective orientation of the crystallites about their long directions it is probably unnecessary to invoke anything more complicated than the simple act of drying.

The authors wish to express their indebtedness to Professor J. H. Priestley for his interest in the work and, together with Miss L. I. Scott, for valuable help on the botanical side. Their thanks are due also to Dr F. C. Steward for a supply of lamellae stripped from fresh cells of *Valonia ventricosa*, and to Mr H. J. Woods for extending the equation of an equiangular spiral described on a sphere to the more general case of a prolate spheroid. For the expenses of the research they are indebted to the generosity of the Worshipful Company of Clothworkers and to the Royal Commissioners of the Exhibition of 1851.

## SUMMARY

The cell wall of *Valonia ventricosa* has been studied in detail by means of X ray diffraction photographs and the polarizing microscope

It is found to consist of layers in which the cellulose chains in any one layer are inclined to those in the preceding and subsequent layers at an angle which is on the average rather less than a right angle

The chains of one set of layers form a system of meridians to the wall, while those of the other set build a system of spirals closing down on the two "poles" defined by the meridians

The two sets of striations on the layers of the wall correspond closely to the meridian and spiral directions of cellulose chains, while the extinction directions, being defined both by the directions and by the relative proportions of the two sets of cellulose chains, lie in variable positions between

The development of the rhizoids has been investigated and found to be associated with regions of the wall adjacent to the poles of the spiral

The plane of spacing 61 Å of the cellulose crystallites is, roughly speaking, confined within an angle of about 60° to the wall surface

It is suggested that the path of the cellulose spiral is that of a logarithmic (equiangular) spiral described on the surface of a sphere or prolate spheroid

The relation is traced between the structure of the walls of fibres of higher plants and that of the cell wall of *Valonia*

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# DESCRIPTION OF PLATES

## Plate 1

- FIG. 2—A typical fringe of fibrils at a torn edge of *Valonia* wall. Note that on entering the wall the fibrils turn round and finally disappear among the striations almost parallel to the edge.  
 FIG. 5—Model of the wall structure of *V. ventricosa* showing the spiral organization of one set of cellulose chains. The spiral can be seen closing in towards the point marked on the model which thus represents one pole.  
 FIG. 6—X ray photograph of the wall of *V. ventricosa* at a pole of the spiral.

## Plate 2

- FIG. 7—Photomicrograph of the basal region of the wall of a cell of *V. ventricosa*.  
 FIG. 8—Photomicrograph of the point of attachment of a rhizoid in cross section. On the right can be seen the watch glass cell from which originated the rhizoid whose remains are attached to the wall on the left.  
 FIG. 9—X ray photograph of an area of the wall of *V. ventricosa* in which one set of cellulose chains greatly predominates (X ray beam perpendicular to the surface).  
 FIG. 10—X ray photograph of the same specimen lying horizontally with the main set of cellulose chains parallel to the X ray beam.



# Development of Eye Colours in *Drosophila*: Pupal Transplants and the Influence of Body Fluid on Vermilion

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## THE PROBLEM

Studies of mosaics (Sturtevant 1932) and transplantation experiments (Ephrussi and Beadle 1935) have shown that the development of vermillion eye colour in *Drosophila melanogaster* is not always autonomous, that is genetically vermillion (*v*) eye tissue may appear phenotypically wild type in the presence of not vermillion tissue in other parts of the body. A specific diffusible substance ' *v*<sup>+</sup> substance', has been assumed to be responsible for this change in the pigmentation of vermillion eye tissue (Beadle and Ephrussi 1936). It is the purpose of this paper to present data bearing on two questions

- 1 When in development is *v*<sup>+</sup> substance produced?
- 2 At what stages of development can the addition of *v*<sup>+</sup> substance produce an effect?

A preliminary discussion of experiments bearing on these questions has been published elsewhere (Ephrussi, Clancy, and Beadle 1936)

## MATERIAL AND METHODS

Three types of experiments have been made

- 1 Transplantation of *v* eye disks from pupae of various ages to wild type larvae, generally within 12 hr of puparium formation
- 2 Transplantation of *v* eye disks from pupae of various ages to wild type pupae of corresponding ages, that is, with no age difference between donor and recipient
- 3 Transfer of body fluid from larvae and pupae of various ages to larvae and pupae of corresponding ages and to larvae and pupae of different ages

All experiments involved the use of material of known ages. Larvae were timed from egg laying. Pupae were timed from puparium formation. In general individuals were collected which had formed puparia within an interval of 1 hr. This interval is convenient because of the fact that after puparium formation darkening of the puparium becomes clear in about 1 hr.

All experiments were carried out at 25° C. Standard mass culture stocks of  $w^a v$  and  $v$  were used throughout. In the second and third types of experiments the wild type flies were  $F_1$  from matings between the two inbred stocks Florida and Swedish  $c$ . The developmental period from puparium formation to eclosion was about 96 hr for the wild type flies and a few hours longer in the  $v$  and  $w^a v$  stocks.\*

The technique of the eye disk transplantation was essentially the same as that described by Beadle and Ephrussi (1936) for larval disks. Transfer of body fluid was made with a small bore pipette without a constriction. In transferring fluid to pupae it was found desirable first to reduce the internal pressure of the recipient by removing a quantity of body fluid approximately equal to the amount to be injected. In general in the body fluid transfers approximately 0.4 cu. mm. of fluid was injected. No special attempt was made to control this amount quantitatively since using pupae as recipients it is impossible with the technique used to be sure that all injected fluid remains in the recipient.

Most individuals injected in the pupal stage failed to emerge normally the injury being of such a nature that the adult flies tended to adhere to the pupal case. In many individuals the pupal case was split and the head protruded. Examinations were made by removing the individuals from their pupal cases at or shortly after the time at which they normally would have emerged.

In all experiments comparable controls were made use of in determining the results. In the transplantation experiments control transplants made between individuals of comparable ages were used. Thus for the transplant to wild type at 55 hr. the two controls  $v$  to  $v$  and wild type to wild type were made at the same time using pupae of 55 hr. In the body fluid

\* To those not familiar with the *Drosophila* work it should be stated that

- (a) The eye colour of wild type flies is dark red.
- (b) The mutant *vermillion* ( $v$ ) is characterized by a bright scarlet eye colour; the locus of the recessive *vermillion* gene is at 33.0 on the first chromosome.
- (c) The recessive *apricot* ( $w^a$ ) is located at 1.5 on the first chromosome; the eye colour of  $w^a$  flies is apricot or pinkish yellow.
- (d) Flies homozygous for both  $w^a$  and  $v$  have eyes of a very pale yellow approaching white.

transfer experiments, controls were made for each stage by withdrawing body fluid from a series of individuals similar to those used as recipients in the experiments and reinjecting it into the same individuals. Such controls like the experimental animals, usually failed to emerge. Controls were made in some experiments by injecting Ringer's solution into individuals similar to those used as recipients. Such controls showed that the operative technique had nothing to do with modification of eye colour and provided standards for determining whether or not fluid from a given stage resulted in any modification of the eye colour of the recipient.

#### TRANSPLANTS OF EYE DISKS FROM VERMILION PUPAE TO WILD TYPE LARVAE

Because of technical difficulties in implanting disks in pupae, the first experiments attempted were made by implanting eye disks from *v* pupae of various known ages into wild type larvae. Such transplants proved to be successful as judged by differentiation of the implants and development of pigment. Disks from pupae at various ages up to 26 hr. after puparium formation gave eyes with wild type pigmentation. When taken from pupae over 26 hr. *v* disks implanted in wild type hosts gave eyes lighter in colour than wild type and approaching *v* controls in appearance. However, control implants from wild type disks taken from pupae over 30 hr. old transplanted to wild type larvae gave eyes with a similar type of pigmentation, that is they were clearly brighter red than standard wild type in wild type controls with no age difference. Since it is known that a genetically wild type eye disk grown in the absence of *v*<sup>+</sup> substance (in a *v* host) after the late larval stage develops wild type pigmentation, it is probable that the *v* like colour developed by 'old in young' wild type control transplants has nothing to do with true *v* pigmentation. Nothing is known as to why or how such control implants develop this *v* like pigmentation, but it is clear that because of this phenomenon, the only conclusion that can be drawn from the experiment is that up to 26 hr. after puparium formation a genetically *v* eye disk when transplanted to wild type host can be modified so as to develop wild type pigmentation.

In an attempt to avoid the difficulty encountered in the experiment just described an essentially similar experiment was made substituting *w*<sup>a</sup> and *w*<sup>a</sup> *v* for wild type and *v*. In this experiment there was no difficulty with controls. Disks taken from *w*<sup>a</sup> *v* pupae at successive intervals up to about 44 hr. and transplanted to *w*<sup>a</sup> larvae developed pigmentation phenotypically close to *w*<sup>a</sup> controls and definitely darker than the *w*<sup>a</sup> *v* controls. Four

series of transplants made after this time gave implants with pigmentation close to  $w^a v$  or intermediate between  $w^a v$  and  $w^a$ . These results can be summarized as follows

Age of implant after puparium formation (hr)	Phenotype of implant
44-46	Intermediate
46-47½	$w^a v$ (?)
50-52	Intermediate
50-52	$w^a v$ (?)

These determinations are not entirely satisfactory in that the results are somewhat inconsistent but they do show that  $w^a v$  disks taken from pupae up to about 44 hr after puparium formation can be modified by a  $w^a$  host in such a way that the colour developed is phenotypically  $w^a$ . Because of possible complications brought about by the age difference it cannot be concluded that after this time a  $w^a v$  eye disk can no longer be changed to  $w^a$  in appearance.

#### TRANSPLANTS OF EYE DISKS FROM VERMILION PUPAE TO WILD TYPE PUPAE OF CORRESPONDING AGES

After the experiments described above were made it was found that successful transplants could be made using pupae as hosts. The results of a series of experiments in which eye disks from  $v$  pupae of various ages were transplanted to wild type hosts are given in Table I. From these data

TABLE I—RESULTS OF TRANSPLANTING VERMILION EYE DISKS TO WILD TYPE PUPAE AT VARIOUS AGES (HOURS AFTER PUPARIUM FORMATION)

Age of donor and host (hr)	Number of individuals	Phenotype of implant
24½-26	3	Wild type
36-37½	2	Wild type
39½-41½	3	Wild type
37-38½	1	Wild type
49-50½	3	Wild type
50-52	3	Wild type
52-54	3	Wild type
54-56	2	Wild type
55-57	3	Wild type
58-59	1	Wild type (?)

it can be seen that up to about 60 hr after puparium formation a  $v$  disk transplanted to a wild type host develops wild type pigmentation. After

this age it was not possible to make transplants because of chitinization of the eyes which at this stage are completely differentiated and, in wild-type flies, partially pigmented. It is seen that the result of this experiment does not agree with that of the one in which  $w^a v$  and  $w^a$  flies were used, presumably because of an unknown effect of the age difference in the one series.

#### TRANSFER OF BODY FLUID FROM WILD TYPE PUPAE TO VERMILION LARVAE AND PUPAE

Preliminary experiments indicated that body fluid from wild type pupae at about 60 hr after puparium formation contains  $v^+$  substance and that  $w^a v$  pupae of the same age are sensitive to this substance. On the basis of this information two sets of experiments were planned: one designed to test for the presence of  $v^+$  substance in the body fluid of wild type pupae at various ages; the other to determine the effect on  $w^a v$  pupae of various ages of injections of body fluid known to contain  $v^+$  substance. Wild type pupae at approximately 60 hr were used as a source of fluid known to contain the substance and  $w^a v$  pupae of the same age were used in tests for the presence of  $v^+$  substance.

Tests for the presence of  $v^+$  substance in the body fluid of wild type larvae and pupae of various ages are given in Table II. It is seen from this table that all tests of late larvae including the use of relatively clear body fluid and of minced whole larvae, gave negative results. Positive tests are recorded for all stages tested between 3 and 80 hr after puparium formation. Tests of all stages older than 80 hr were negative. Unfortunately, as already stated, it was not found possible to control quantitatively the amount of fluid transferred. However, a rough idea of the amount of substance is presumably indicated by the proportion of positive effects among the test animals. Comparisons at various stages suggest that the amount of  $v^+$  substance increases rapidly, beginning shortly after puparium formation, reaches a maximum at a period between 8 and 17 hr, and then shows a gradual decrease up to 80 hr after which time no positive tests were obtained. It should be noted that the strongest modifications among the positive tests in this series were observed in the 8½–10½ hr tests.

Tests for the influence of injected fluid known to contain  $v^+$  substance (from wild type pupae used at about 60 hr) are summarized in Table III. The data show that larvae shortly before puparium formation give positive tests. Since positive tests were found for 60 hr pupae (Table II), it is assumed that all stages between puparium formation and 60 hr thereafter

TABLE II—RESULTS OF INJECTING BODY FLUID FROM WILD-TYPE LARVAE AND PUPAE OF VARIOUS AGES (HOURS AFTER PUPARIUM FORMATION) INTO APRICOT VERMILION PUPAE AT APPROXIMATELY 60 HR AFTER PUPARIUM FORMATION OF THE RECIPIENTS

Age of donors (hr)	Age of recipients (hr)	Recorded effect on recipient			Total tests
		Positive	Questionable	Negative	
Late larvae*	Late larvae	0	0	5	5
Late larvae	57½-59½	0	0	10	10
Late larvae	58½-59½	0	0	12	12
Late larvae†	59-60½	0	0	9	9
3-5	59-60½	7	0	13	20
4½-6	60½-68	0	4	7	11
8½-10½	54½-56½	13	3	1	17
12-13½	62½-64	12	2	4	18
15½-17	62½-64	9	1	3	13
16-17½	59½-61	7	5	4	16
20-21½	60½-62	6	4	8	18
24½-26½	59-61	8	4	6	18
41-43	62-64	8	9	2	19
56½-58½	56½-58½	3	1	5	9
58½-60½	58½-60½	2	0	5	7
69½-71	55-56½	1	1	17	19
74-75½	51-52½	4	3	12	19
79-80½	59½-60½	1	7	3	11
81-82½	59-60½	0	0	9	9
84-85½	60-62	0	0	16	16
90-91½	58-59½	0	0	19	19
90½-91½	50½-52	0	0	12	12
94½-96	58-59½	0	0	15	15

\* Cinnabar larvae used as source of body fluid in this experiment

† In this experiment donors were minced, and fluid drawn off with pipette

TABLE III—RESULTS OF INJECTING BODY FLUID FROM WILD-TYPE PUPAE OF APPROXIMATELY 60 HR AFTER PUPARIUM FORMATION INTO VERMILION LARVAE AND PUPAE OF VARIOUS AGES

Age of donors (hr)	Age of recipients (hr)	Recorded effect of recipient			Total tests
		Positive	Questionable	Negative	
59½-61½	Late larvae	2	2	0	4
59½-61½	69-70½	0	0	20	20
59½-61½	75-76½	0	0	16	16
60-61½	81½-82½	1*	1	19	21
59-60½	84½-86	1*	4	15	20
58½-59½	89-90½	0	3	8	11
60½-62	94½-95½	0	0	7	7

Weak positive tests

likewise would be sensitive to injected fluid. Positive tests are indicated in Table II for 62–64 hr pupae. Pupae 69–70 hr old gave either negative or weak positive tests indicating that after this time the addition of  $v^+$  substance is able to produce relatively little or no change.

It is probably useless to attempt to draw any conclusions concerning differences in sensitivity to a given amount of  $v^+$  substance of pupae of various ages from the data available. Larvae give strong positive tests but it is certain that more injected fluid actually remained in the animals at this stage. In late pupal stages after about 80 hr the internal pressure is rapidly reduced and presumably larger amounts of body fluid remained in the animals. This may account for the fact that no positive tests were obtained from 69 to 77 hr while weak effects were recorded from 81 to 86 hr.

#### DISCUSSION

From the foregoing results it is clear that fluid from wild type prepupae and pupae over a rather wide range of ages (3–80 hr after puparium formation) is capable of producing an effect on the eye colour when injected into  $w^a v$  recipients. It has been assumed that this effect is brought about because of the fact that the body fluid contains  $v^+$  substance. However since the crude body fluid used certainly contains many living cells it is possible that the injected cells produce the substance at an unknown time after the injections. The fact that fluid from larvae which also contains many living cells does not produce such an effect makes this assumption rather improbable. Obviously a direct test of whether or not living cells have anything to do with the effect can be made: this has not yet been done.

The experimental evidence presented above indicates that normally the  $v^+$  substance must be taken up by the eye (not necessarily used) before 65 to 70 hr after puparium formation since after that time it is no longer present (or is present in only small amounts) in the body fluid. Larvae at a stage shortly before pupation show positive effects of injected body fluid containing  $v$  substance but since the effect is observed only in the mature fly it cannot be determined from the available information when this injected  $v^+$  substance acts on the eye. It is entirely probable that it may remain in the body fluid and be used by the eye many hours later.

Until further information is available it is useless to consider questions concerning the nature of  $v^+$  substance whether for example it is of the nature of a hormone or is a precursor which actually takes part in the reactions leading to the formation of specific type of eye pigment.

## SUMMARY

Transplants of *v* eye disks to wild type hosts show that a genetically *v* eye disk can develop wild type pigmentation after transplantation at as late a stage of development as that reached 55-60 hr after puparium formation (25° C)

Body fluid transfers show that a substance (*v*<sup>+</sup> substance) capable of producing a change in the pigmentation of the normal eyes of a *w*<sup>+</sup> recipient is present (or can be produced by some component of the body fluid) in wild type flies from about 3 to 80 hr after puparium formation. Before and after this period this substance either is absent or is present in such low concentrations that tests for its presence were negative. Flies of the genetic constitution *w*<sup>+</sup>*v* show a positive response to the injection of body fluid known to contain *v*<sup>+</sup> substance from the late larval stage (possibly before) up to about 65 hr after puparium formation. After this time the responses were negative or weak.

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# Synaptic Transmission of Nervous Impulses through the last Abdominal Ganglion of the Cockroach

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[Plate 3]

In a previous communication (1936) we have described the response of the cercal nerve of the cricket when the cercus is subjected to acoustic stimuli. In the course of that work we attempted to trace the afferent fibres to their destination by recording from the ventral nerve cord at various levels anterior to the last abdominal ganglion. It was immediately apparent that while some of the fibres from the acoustically sensitive end organs of the cercus ran directly through the ganglion and up the cord others terminated in the ganglion in synaptic relation with a relatively small number of fibres running forwards in the cord and yielding action potentials of considerable magnitude. Contrary to expectation we found that subject to certain conditions noted below the random activity in the abdominal nerve cord was never large enough to obscure the wanted signals and it seemed to us that the preparation offered an excellent opportunity for an examination of the properties of a central nervous synapse. This paper describes the results of this examination.

## THE PREPARATION

In this work we have used the American cockroach (*Periplaneta americana*). As in the previous work (1936) the posterior part of the nervous system of a decapitated animal was exposed by dissection from the ventral aspect. Electrical activity in the cord which tended to mask the effects which we wished to record seemed to arise principally from two causes: (a) descending impulses originating in the thoracic ganglia and (b) injury discharges. The former could be blocked by section or ligation of the cord anterior to the first abdominal ganglion. They appeared however to arise

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in connexion with sensory impulses from end-organs in the legs and were generally absent when the latter were amputated close to the body. Injury discharges resulted from tension on the nervous system in dissection, from desiccation, and from extensive injury to the tracheal supply. With practice in dissection and the use of an animal which contained plenty of blood and was mounted in a moist chamber we experienced very little trouble from this cause.

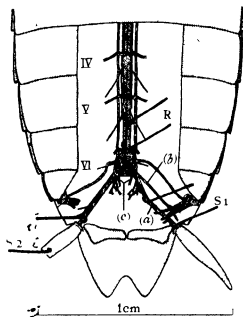


FIG. 1.—Sketch of posterior part of the abdominal nerve cord of cockroach (♀) with the principal tracheae and the positions of stimulating and recording electrodes. The external genitalia have been removed. *R* = recording electrodes. *S*<sub>1</sub>, *S*<sub>2</sub> = alternative positions of electrodes for stimulating afferent cercal nerve. (*a*) cercal nerve (sensory), (*b*) cercal nerve (motor), (*c*) nerves to genitalia (cut). IV, V, VI = last three abdominal ganglia.

Fig. 1 indicates the general relations of the nervous system in the lower part of the abdomen and the positions of stimulating and recording electrodes. For the latter we have found gold wire (0.005 in. diam.) preferable to platinum as, especially after heating and quenching, it is inelastic and can be pushed and bent into any desired form.

## THE APPARATUS

The preparation together with its recording electrode system is installed in a sound proof electrostatically shielded room. Stimulation either acoustical of the anal *cercus* or electrical of any appropriate nerve has been arranged respectively by means of a loud speaker or of a separate pair of stimulating electrodes. These instruments constitute the remainder of the apparatus in the sound proof room all apparatus for the generation of stimuli and for the recording of responses being situated in a room entirely separate though closely adjacent to the latter.

The apparatus used for sound stimulation consists of a heterodyne oscillator coupled through an amplifier to the loud speaker referred to. Attenuating networks enable control of the intensity of the stimulus over a range of 140 db in 2 db steps and the oscillator is arranged to deliver approximately sinusoidal voltages at any frequency between 30 and 11 000 c/sec. A more complete description of this section of the apparatus may be found under the references given (Rawdon Smith 1935 1936).

For electrical stimulation we have used a double gas discharge tube circuit so arranged that either one or both of the tubes might be employed in this way permitting the interpolation of rare stimuli from one tube into the regular series derived from the other. This instrument which is conventional in circuit is coupled by means of a special transforming device to the stimulating electrodes. Details of this will be found in an earlier paper (Pumphrey and Rawdon Smith 1936 in Press) and it will suffice here to say that it consists of a fixed electrostatically shielded secondary winding connected to the stimulating electrode leads and a movable primary winding inserted in both gas discharge tube circuits. Movement of the primary relative to the secondary permits ready control of the magnitude of the stimulus without alteration of its wave form a prominent disadvantage of the more normal potentiometric intensity control for such a stimulating circuit. In our own case the wave form\* of the stimulus is diphasic in virtue of the inductive coupling referred to is such as to reduce to a minimum polarization effects at the stimulating surfaces. The importance of this may be noted later.

For recording purposes we have employed two identical resistance capacity coupled triode amplifiers of variable gain. The input circuits of these consist of the arrangement described by Matthews (1934) earth free

\* An additional advantage of this arrangement is that the duration of the stimulus is very short (approximately 0.5 msec). For this reason the stimulus artefact rarely obscures the response even for very small interelectrode distances.

input leads being of great advantage when using electrical stimuli and essential when recording simultaneously from more than one point on the same animal. By the selection of suitable valves it has proved possible to abolish the resistance capacity high frequency oscillation suppressors there described with some improvement of the high note response of the unit. Each input circuit and amplifier may be finally connected to a Cossor 3236 J cathode ray oscillograph and either or both of these may be photographed on moving ciné bromide paper by means of an adapted film camera. Either or both recording systems may be used at a time or if necessary one may be used for recording and the other for observation. The latter is assisted by a linear sweep device which may be synchronized either to the stimulus or the response voltages. A description of this has been given by one of us elsewhere (Hallpike and Rawdon Smith 1934).

## RESULTS

### 1—Arrangement of Fibres in last Abdominal Ganglion

Before describing our experiments in detail we shall indicate the arrangement of pre and post ganglionic fibres so far as we have been able to elucidate it. \* The cercal nerve proper which may be separated from the small motor branch to the cercal muscles accompanying it over part of its course contains only afferent fibres. Some of these run through the abdominal ganglion into the ventral nerve cord. Stimulation either auditory of the cercus or electrical of the cercal nerve elicits a response in the cord above the last ganglion in addition to the major response discussed below similar to that in the cercal nerve when due allowance is made for the greater scatter due to the increased conduction distance and the diminished amplitude due both to this factor and to the short circuiting effect of the other fibres in the cord. Our experiments indicate that most of these fibres which we shall term *through fibres* run on the same side of the cord as the cercus from which they are derived. We have not been able to trace them with certainty to higher levels of the cord.

Stimulation of the cercal nerve of one side however in addition gives rise to post ganglionic impulses of great magnitude in fibres on both sides of the cord though there are relatively fewer of these on the *contra lateral* side. These post ganglionic fibres we shall term *giant fibres* from their resemblance to fibres so named in other invertebrates. By recording

No histological examination has yet been made of the last abdominal ganglion in any Orthopteran. Our conclusions are not inconsistent with the arrangement of ganglion cells found in *Aeschna* by Zawarzin (1924).

simultaneously from different points on the cord, we have shown that they run directly through the next five abdominal ganglia and impulses in most, if not certainly in all of them, can be detected in the neck just posterior to the sub oesophageal ganglion. The arrangement which we have tentatively assumed for these fibres is indicated in the diagram (fig 2). The through fibres may be differentiated with certainty from those synapsing in the ganglion by interchanging the stimulating and recording electrodes. A stimulus then applied to the cord will reveal, in the cercal nerve, only a minor response due to the through fibres, antidromic stimulation of the giant fibres producing no effect there.

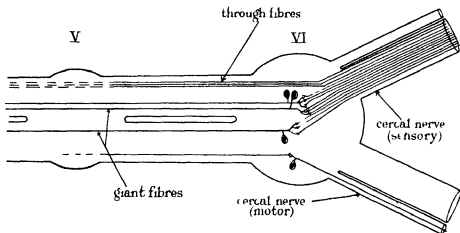


FIG 2

### 2—Acoustic Stimulation of the Cercus

It might be thought that, for the investigation of the response of the giant fibres to pre-ganglionic activity, acoustic stimulation of the end organs associated with the peripheral afferent fibres would be very satisfactory, since the frequency and intensity of the stimulus could be varied with great ease and precision and, more important, one might be certain that only fibres homologous in that they are all derived from acoustically sensitive sensilla were stimulated. But synchrony of response in the cercal nerve is very imperfect at the lowest frequencies of auditory stimulation (especially in the cockroach) as some of the hair sensilla are excited more than once by each incident wave of the stimulus. For this reason we have abandoned acoustic in favour of electrical stimulation of the peripheral nerve. It is worthy of note, however, that in the *cricket* where synchrony of the response in the cercal nerve to auditory stimuli is more nearly perfect

we have obtained a giant fibre response synchronized with the stimulus at frequencies between 70 and 100 c/sec, and momentarily at frequencies considerably higher than this

### *3—Response to a Single Electrical Stimulus of the Cerecal Nerve*

When the cercal nerve is stimulated by a single maximal shock and the recording electrodes are on the cord above the last ganglion, the stimulus is followed almost immediately by a response from the through fibres and, after a pause, by a closely grouped volley of impulses in the giant fibres (fig 3, Plate 3) The pause consists in part of the conduction time and in part of the delay at the synapse By varying the positions of the electrodes we have estimated the conduction rate in pre- and post-ganglionic fibres as approximately 1.5 and 5–6 m/sec respectively and from this deduced a synaptic delay of about 1 msec \*

Under optimal conditions, as has been stated above, we have generally experienced little trouble from intrinsic and apparently spontaneous activity in the cord Occasionally, however, we have observed a good deal of activity which was not associated with movements of the thorax and did not possess the very characteristic features of an injury discharge We are inclined to regard this activity as a consequence of operative shock rather than a normal condition of the abdominal central nervous system, since it died down spontaneously if we waited We mention it here, however, because in this hyperexcitable condition the post-ganglionic volley consequent on stimulation of the cercal nerve was very frequently followed by a pronounced after-discharge, apparently in the same fibres

Reduction of the strength of the stimulus results in a progressive reduction of the number of giant fibres which respond and by careful adjustment it is generally possible to obtain a discharge in a single giant fibre (fig 4, Plate 3) At intensities which are just supraliminal,† however, it is usually impossible to get a regular response to successive stimuli, owing probably to intrinsic variations in the thresholds of pre-ganglionic fibres We have successfully reduced the number of active giant fibres to one by

\* Because of the small size of the preparation, the accuracy of these determinations is probably low By making extreme allowance for errors of observation we estimate the synaptic delay to be not less than 0.4 nor more than 2 msec for a single pre-ganglionic stimulus

† Terms such as this have reference to the effect of the stimulus on the post-ganglionic fibres, e.g. a just supraliminal stimulus is one which when applied to the pre-ganglionic nerve will sometimes produce a small giant fibre response Similarly a maximal stimulus is one such that an increase in the stimulus intensity produces no increment in the giant fibre response

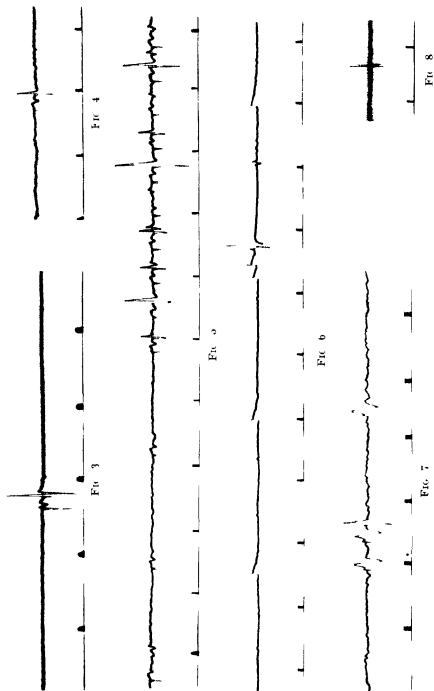
dissection of the cord, but injury to the cord seems to be progressive and the life of such a single fibre preparation has so far proved too short for experiment

#### 4—Responses to Repetitive Stimulation

When the pre-ganglionic fibres are subjected to repeated maximal or supramaximal stimuli at regular intervals, each volley of the giant fibre response is *approximately* identical, provided that the stimulating frequency is low. The small variations in the grouping of the giant fibre responses we take to be an indication that the delay for each synapse is not absolutely invariant even at low frequencies of stimulation. Above a certain frequency of stimulation, there is a falling off in the total number of fibres responding to each stimulus, and, as the number of active fibres is reduced, it can be seen that the response of the remainder becomes intermittent before failing totally. At this stage it may also be seen that the interval between stimulus and response becomes somewhat longer and more variable. This increase in the interval, which may be 1–2 msec, is attributable to alteration in the synaptic delay, since the frequencies employed are not such as would cause an increase in the conduction time of either pre- or post-ganglionic fibres.

When repetitive submaximal stimuli are applied to the cercal nerve, the post-ganglionic response suffers a decline in magnitude, provided that the stimulus frequency is sufficiently high. At any given stimulus intensity, however, it is possible to find a frequency which just fails to cause such a decline. This value we have called the *critical frequency*, and it is clear that it is not the same for all intensities of stimulus. In our preparation, it varies from 40 to 50 per sec. for just maximal stimuli to 15 per sec. or less for submaximal. If repetitive stimuli of submaximal intensity are applied at a frequency above the critical, the giant fibre response eventually disappears almost completely. An increase in the stimulating intensity will now bring back the response, as would be expected. By recording pre-ganglionically we have satisfied ourselves that this effect is due to an increase in the number of pre-synaptic fibres excited, though there is no sign of a decrease in this number during the time when the post-synaptic response is decaying.

It is not, however, necessary to increase the intensity of the stimulus to recover the post-synaptic response, for an increase in the stimulating frequency, without modification of the intensity, is temporarily as efficacious (fig. 5, Plate 3). Investigation of the pre-ganglionic response under these conditions reveals that there is no change whatever in the amplitude or form of the action potential here, and that the only modification of this







response is an increase in frequency. It appears justifiable, therefore, to regard this augmenting effect of increased stimulus frequency as having its locus in the synaptic connexions between the cercal fibres and the giant fibres of the ventral cord.

It would be deduced from the foregoing data that, during repeated stimulation, when the post-synaptic response has become almost negligible, the interpolation of an extra stimulus into those of the regular series should cause a momentary return of the response. This expectation has been found to be justified and, in fig. 6, Plate 3, it may be seen that such an additional stimulus, even though its magnitude is considerably smaller than those of the series proper, is efficacious in causing a single response to the stimulus of the regular series immediately following it. On occasions, the response secured in this way may occur to the interpolated stimulus itself (fig. 7, Plate 3), especially if this should occur immediately after one from the series. The relative sizes of the two stimuli may be noted from fig. 8, Plate 3.

In fig. 7, Plate 3, it is clearly seen that the response of the through fibres, which do not synapse in the last abdominal ganglion, is maintained throughout the experiment. In this figure the through fibre response is, relative to that from the giant fibres, somewhat greater than is customary, and it may be worthy of note that the relative sizes of the two appear to be to some extent dependent on the age of the preparation.

In all these cases it is often possible to identify, in the oscillograph figure, the responses of single fibres which, owing perhaps to some special relation to the recording electrodes, display an action potential wave of characteristic form. It is thus possible to be certain that a response which has disappeared, after repeated stimulation, reappears in the same giant fibre on raising either the frequency or the intensity of the stimulus. It follows, therefore, that some at least of the giant fibres in this insect are synaptically connected with two or more pre-ganglionic fibres.

It might also be expected that a preparation which behaved in this way would display the phenomena of temporal summation in the classical sense. This expectation is not fulfilled. It is impossible to find a frequency and intensity of stimulus such that the initial stimuli of a series are ineffective in producing a post-ganglionic response while later stimuli are successful. Indeed, it may be shown that, when a series of liminal stimuli is applied to the preparation, the response is erratic in that by no means every pre-synaptic stimulus yields a post-synaptic response. The first stimulus of the series, however, is as likely to yield a response as any other. This appears to be true irrespective of the stimulation frequency. Moreover, we have

not been able to demonstrate a shortening of the synaptic delay for a stimulus following rapidly on another such as has been described for the mammalian flexor reflex (Eccles and Sherrington 1931)

Both these considerations argue against a slow building up of the excitatory state and the existence of a subliminal fringe. It is clear that current theories of central excitation are not directly applicable to our preparation

#### DISCUSSION

Repeated maximal stimulation of the cercal nerve at a frequency above the critical frequency results in the giant fibres no longer being excitable through their synapses though both pre and post ganglionic fibres may still be excited by direct electrical stimulation. This condition of the synapses we have designated *fatigue* since it appears to be in every way comparable with the fatigue of the neural end plates in vertebrate striated muscle

It is of interest to contrast these observations with those of Bronk and Pumphrey (1935) on the synapses of the inferior cervical ganglion of the cat. They found that stimulation of the upper thoracic sympathetic roots at a low frequency produces a synchronized discharge in the post ganglionic fibres of the inferior cardiac nerve. At higher stimulation frequencies (say 80/sec) the synchronous volleys disappear after the first few stimuli and thereafter (with monophasic leads and a direct coupled amplifier) the post ganglionic response is seen to consist of a smooth negative deflexion of the base line co durational with the period of stimulation. It was shown that pre ganglionic stimulation was effective in maintaining the acceleration of the heart for long periods at stimulus frequencies from 60 to 120 per sec and that within this range the degree of acceleration was independent of the frequency. It was concluded that at the higher frequencies the post ganglionic fibres entered into a self regulatory cycle of excited and refractory phases independently of each other and of the periodicity of the incoming pre ganglionic impulses producing a random discharge in the post ganglionic trunk of which the negative deflexion of the base line represented the statistical sum. This preparation therefore did not show *fatigue* of the type which we find in the cockroach.

With repetitive submaximal stimulation the situation is more complex and our results invite comparison on the one hand with those obtained from the study of mammalian reflexes and on the other with observations of the physiology of the elementary nerve net of the Actinozoa.

Repetitive submaximal stimulation of the cercal nerve gives rise to a condition of the synapses which we have designated *adaptation*. It has been noted that there is a fall in magnitude of the response with time during a period of repetitive stimulation at a constant intensity but this does not prevent the preparation from responding to a change in the magnitude of the stimulus. We believe the analogy with the adaptation of sensory end organs to be very close (cf. for example Bronk and Stella 1935).

In the adapted condition the preparation responds by an augmentation of the post ganglionic response not only to an increase in the intensity of the stimulus but to an increase in frequency. The latter type of augmentation is clearly allied to the phenomenon of summation which is a widely distributed property of synapses (Sherrington 1906, Pantin 1935). It must be emphasized however that in our preparation summation occurs only in the adapted state. In the unadapted synapse it is quite impossible to obtain a response from a series of stimuli of such a magnitude that each alone is subliminal. As has been pointed out the response is irregular at stimulus intensities just on the threshold but whatever the frequency the response if it occurs at all is as likely to occur to the first stimulus of a series as to any other. There is here no parallel with the phenomena of summation described by Sherrington (1906).

We believe that the explanation of these facts is as follows: the arrival at the *unadapted* synapse of an impulse along a *single* pre ganglionic fibre is adequate to initiate an impulse in the corresponding post ganglionic fibre. In support of this view we find firstly that on gradually reducing the intensity of a very slow series of stimuli the pre and post ganglionic responses disappear completely at the same intensity and secondly that stimulation of a single pre ganglionic fibre by agitation of the hair sensillum from which it is derived is often adequate to produce a post ganglionic response.

It seems probable therefore that adaptation of the synapse consists in a prolongation of the relatively refractory state of the synaptic terminations of pre ganglionic fibres. It may be supposed that the arrival of an impulse at a synapse from a single pre ganglionic fibre raises what we may call the excitatory potential whether by causing the secretion of a neuro humor or otherwise. In the unadapted synapse the potential reaches the threshold of the post ganglionic fibre and initiates an impulse in the latter. In the adapted synapse the threshold is raised so that an impulse in a single afferent fibre fails to excite: the excitatory potential can be raised to the higher threshold by exciting more pre ganglionic fibres or by decreasing sufficiently the interval between successive stimuli. It must therefore be

assumed that the complete decay of the excitatory potential occupies a time longer than the interval between stimuli at the frequencies at which summation occurs in the adapted preparation (i.e. at least 20 msec). On the other hand we have reason to believe that it reaches its maximum relatively rapidly since the synaptic delay is never longer than 3-4 msec even in the fatigued or adapted preparation.

It must also be assumed that if stimulation of one pre-ganglionic fibre raises the excitatory potential at the synapse to the threshold the simultaneous stimulation of several will raise it much higher. There is therefore a possibility of re-excitation of the post-ganglionic fibres when they have passed through their refractory phase. This we believe to be the most probable explanation of the after-discharge sometimes observed (p. 111).

The fact that summation is demonstrable in the adapted synapse also renders it unlikely that the initiation of an impulse in a post-ganglionic fibre uses up an appreciable amount of the excitatory potential.

In our discussion we have purposely avoided extending the term *oes* to cover our observations. This expression has already become somewhat ambiguous through its application to the phenomena observable in peripheral ganglia and it seemed advisable to describe our results so far as possible in terms such as fatigue and adaptation which are already in spite of difficulties in definition well recognized phenomena in the physiology of the periphery. We have used the expression *excitatory potential* because while its meaning is self-evident it has no historic associations and implications. As has been shown it has some attributes in common with *oes* and others which are not.

The facilitatory effect of interpolating a single extra stimulus into those of a regular series may be compared with certain phenomena to be noted in human subjects\*. If a series of regular short duration acoustic stimuli is applied to such a subject at an intensity sufficient to elicit the blink reflex response it will be found that under certain conditions of duration intensity and spacing of the stimuli the response gradually dies away. It is then said to be *inhibited*. If now a stimulus of similar type is interpolated in the series a response will be obtained occasionally to the interpolated (*disinhibitory*) stimulus itself though more often to that of the regular series immediately following it. Precisely similar phenomena as has been noted are seen in our own case.

Attention may also be drawn to the somewhat less obvious though none the less clear similarity between these results and certain other effects

\* We are greatly indebted to Mr R. C. Oldfield for this information and for his permission to use it here.

obtained in human subjects (Rawdon Smith 1936) It was here shown that the application of a sustained monaural pure tone stimulus of considerable intensity led to a loss of auditory sensitivity both ipse and contralaterally The ensuing application of an unexpected stimulus in the same or another sensory mode led to a recovery of contra lateral sensitivity for a period of several seconds

#### SUMMARY

A method is described whereby the response in the central nerve cord to either electrical stimulation of the cercal nerve or acoustic of the cercus itself may be investigated in the cockroach

Investigation reveals that the response in the cord anterior to the last ganglion is twofold first, a small response due it is thought, to the presence of a small number of fibres which pass through the ganglion without synapsing and which have been called, therefore, 'through fibres' and secondly a response of great magnitude due to what have been called 'giant fibres' The latter, it is contended, are few in number and there is evidence that each such fibre is in synaptic connexion with a number of fibres from the cercal nerve

The post ganglionic response to repetitive submaximal electrical stimuli applied to the cercal nerve exhibits a number of interesting features At low stimulus frequencies (around 25/sec) the response exhibits a decline in amplitude, until the majority of the stimuli fail to excite the post ganglionic fibres, though it is shown that the pre ganglionic response is in every way identical throughout the stimulating period The post ganglionic response may be brought back in one of three ways by increasing the number of peripheral fibres stimulated, i.e. raising the stimulus intensity by increasing the stimulus frequency, without modification of its intensity or by the interpolation of an extra stimulus into the series Such a stimulus yields only a momentary effect, but nevertheless produces unmistakable results even if it is of much smaller intensity than the stimuli of the series in which it is inserted

A tentative theory is put forward to account for these phenomena

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#### DESCRIPTION OF PLATE

All records read from left to right Time in all cases = 20 msec

- FIG 3—Post ganglionic response to single maximal pre ganglionic stimulus Note from left to right (a) stimulus artefact (b) response of through fibres (c) large response of giant fibres
- FIG 4—Post ganglionic response reduced to single giant fibre note persistence of through fibres
- FIG 5—Post ganglionic response to repetitive pre ganglionic stimulation Note that the slow rate of stimulation approx 28/sec fails to produce a post ganglionic response but that when the stimulating rate is raised to approx 105/sec a response is readily secured though the stimulating intensity is identical in both cases
- FIG 6—Post ganglionic response to repetitive pre ganglionic stimulation Stimulation at 27/sec fails to produce a response until the interpolated stimulus is applied A large response is then secured to the next stimulus of the series
- FIG 7—Similar to fig 6 though another preparation On this occasion the interpolated stimulus coming shortly after one from the regular series itself yields a response Note the persistence of the through fibres which respond to each stimulus
- FIG 8—The relative sizes of the two stimuli used in figs 6 and 7 The smaller of the two is the interpolated stimulus
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## On the Haematin Compound of Peroxidase

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It was shown previously (Keilin 1927) that horseradish roots contain a large concentration of haematin which can easily be seen spectroscopically as pyridine-haemochromogen in slices of roots 5–10 mm thick which have been treated with pyridine and  $\text{Na}_2\text{S}_2\text{O}_4$ .

Similar haematin compounds are widely if not universally distributed in cells and tissues of all aerobic organisms where they can be observed in addition to or in absence of the haematin of cytochrome (Keilin 1925).

In 1931 Kuhn, Hand and Florkin claimed to have found a close parallelism between the enzymic activity of horseradish peroxidase preparations and the total haematin content of these preparations estimated as pyridine-haemochromogen. They found, for instance, that in their three enzyme preparations of P Z (purpurogalline number), † 1080, 1710 and 3400, the concentrations of haematin were respectively 0.038, 0.060 and 0.105% of the dry weight of the preparations. These results have led them to the conclusion that the enzyme is probably a haematin compound.

Elliott and Keilin (1934) reinvestigated the problem, but did not find a strict proportionality between the enzyme activity and the total haematin content of various peroxidase preparations obtained by different methods. They also found that the concentration of haematin in these preparations was much higher than that observed by Kuhn *et al.* (1931). Thus a preparation of P Z 818 contained 1.05% of haematin, while Kuhn, Hand and Florkin found only 0.105% of haematin in their preparation which they claim to be of P Z 3400. Such a discrepancy could hardly be explained by a natural break in the proportionality which may occur during purification.

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† Purpurogalline number throughout our work is expressed as mg. of purpurogalline formed from pyrogallol in 5 min. at 20° C. per 1 mg. of dry weight of peroxidase preparation. The estimation is carried out in presence of 12.5 mg. of  $\text{H}_2\text{O}_2$  and 1.25 g. of pyrogallol in 500 c.c. of water. The concentration of enzyme is so adjusted that no more than 10 mg. of purpurogalline is formed during the experiment.



tion of the enzyme and is most probably due to some errors in their determinations. In the light of these results Elliott and Keilin discussed several possibilities as to the relationship between the haematin and the enzyme. They did not arrive, however, at any definite conclusion and left the problem open for further investigations.

While investigating the distribution of haematin compounds in plants we had recently an opportunity of returning to the problem of the haematin nature of peroxidase. We have confirmed the results obtained by Elliott and Keilin as to the total haematin concentrations in different enzyme preparations and we have again found the break in proportionality between the enzyme activities and the concentration of total haematin.

By approaching the study of this problem from another angle, however, we are now able to bring forward new and more reliable evidence supporting the view that the active group of peroxidase is a haematin compound. We are able to describe for the first time the properties of this haematin in relation to those of other and better known haematin compounds. These results were obtained in spite of the fact that the breaks in proportionality between the enzyme activity and the concentration of total haematin do occur during the purification of the enzyme. Moreover, we hope to show that the existence of these breaks does not necessarily contradict our conclusions.

#### MATERIAL AND METHODS

The peroxidase used for this investigation was prepared from the roots of non cultivated horseradish essentially by the method of Elliott (1932).

7 kg. horseradish, washed and cut into small pieces, were minced twice in a strong gr. and minced and extracted twice with 6 l. of water. The mixture pressed out with a hydraulic press gave about 14 l. of crude brownish extract (P Z 0.65). The extract was saturated with ammonium sulphate giving a greyish precipitate which gradually aggregated and rose to the surface. The mixture was filtered on a Buchner filter: the precipitate suspended in about 400 c.c. of water and the mixture then dialysed in a long cellophane tube against running tap water. After about 48 hr. the fluid was almost completely free from ammonium sulphate and it was then dialysed for a short period against distilled water. About 600 c.c. of this fluid were mixed with 1200 c.c. of alcohol (90%). The resulting precipitate was removed by centrifuging giving 8 g. (dry weight) having P Z 14. The remaining fluid (1700 c.c.) was mixed with 1200 c.c. absolute alcohol. The precipitate obtained was centrifuged off and gave 1.3 g. having P Z 160.

Other preparations of horseradish gave very similar results. The P Z of the first fraction varied from 14 to 26 and that of the second fraction from 112 to 300. Practically all the properties of peroxidase haematin can be easily determined in the second fraction of this preparation without any further purification. We have used, however, for some of our experiments preparations of much higher activity which we

have obtained from the second fraction. For instance, 1 g. of peroxidase preparation of P Z 270, dissolved in water and reprecipitated twice with alcohol, was redissolved in water and mixed with 100 mg. of freshly prepared tricalcium phosphate gel. The brown coloured gel when centrifuged off was found to be almost free from peroxidase. Precipitation of the solution with alcohol gave 215 mg. of P Z 550. Residues from the above purification were worked up to give 210 mg. of P Z 165. 140 mg. of P Z 550 redissolved in 50% alcohol was purified by a single adsorption on alumina *A* (prepared and used according to Grassmann (1928)) and gave 70 mg. of P Z 1000. One precipitation by tannic acid and one adsorption on alumina *A* followed by a concentration of the elution at room temperature raised the activity of this preparation to P Z 1500.

The concentration of the total haematin in various preparations and in horseradish root itself was determined as pyridine haemochromogen by the method previously described (Elliott and Keilin 1934). The estimation of the correct positions of the absorption bands was carried out by means of a Hartridge reversion spectroscope adapted to a microscope.

#### PEROXIDASE HAEMATIN

It was shown previously (Elliott and Keilin 1934) that a strong peroxidase preparation has an absorption spectrum composed of three bands, a strong band in the red at  $642.5\text{m}\mu$  and two other bands at 550 and  $500\text{m}\mu$  which are partly masked by the general absorption in this region of the spectrum due probably to another yellow pigment present in the prepara-

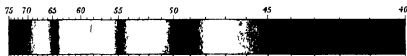


Fig. 1—Absorption spectrum of peroxidase

tion\*. This absorption spectrum was previously ascribed by Elliott and Keilin to acid haematin to which it shows a great resemblance. The study of the properties of this compound made on fresh material has convinced us now that the absorption spectrum of peroxidase preparation is not that of an acid haematin but a compound of the methaemoglobin type very similar to catalase. This investigation was greatly helped by some of the

\* The absorption band at 630 or  $640\text{m}\mu$  seen by Sumner and Howell (1936) in peroxidase preparations from fig sap corresponds to the first band of the absorption spectrum described by Elliott and Keilin (1934). However, contrary to the statement by Sumner and Howell this band like the corresponding band of acid methaemoglobin disappears on making the solution slightly alkaline (pH 10). The haematin content in their preparation of P Z 700 was found by them to be a little higher than 1%, which is in agreement with the results obtained by Elliott and Keilin.

results obtained recently in the study of methaemoglobin and of the catalase haematin (Keilin and Hartree 1935, 1936).

The absorption spectrum of peroxidase haematin compound is very well seen in a clear solution of 10 mg of peroxidase of P Z 100 or more dissolved in 0.5-1 c.c. of water and examined in a layer 1-2 cm deep. It consists of four absorption bands the correct positions of which, when measured with a Hartridge reversion spectroscope, are slightly different from those given in our previous paper (Elliott and Keilin 1934). They are I, 645  $m\mu$ , II, 583  $m\mu$ , III, 548  $m\mu$ , and IV, 498  $m\mu$ . The fourth band is probably the strongest, but the first band in the red, not being masked by the absorptions of other pigments, is usually the most conspicuous and can easily be seen in preparations of a much lower activity and even in horse radish root itself. The second band (583  $m\mu$ ) is very faint and hardly perceptible in preparations of low activity, so that for all practical purposes the absorption spectrum of peroxidase haematin can be considered to be composed of three bands only. That this absorption spectrum belongs to a natural thermolabile protein combined with haematin is clearly demonstrated by its irreversible disappearance on boiling or by treatment with excess of acid or alkali.

On the other hand, the haematin of strong peroxidase preparation is easily converted into protohaemin or Teichmann's crystals. The latter dissolved in pyridine and treated with  $\text{Na}_2\text{S}_2\text{O}_4$  gives a typical pyridine-protohaemochromogen (Elliott and Keilin 1934). This clearly shows that the haematin nucleus in peroxidase is protohaematin probably identical with that of blood haemoglobin.

#### ALKALINE PEROXIDASE HAEMATIN

On neutralizing the solution of peroxidase and making it slightly alkaline the absorption spectrum undergoes a gradual modification which begins at pH 9 and is complete at pH 11. The three-banded absorption spectrum is replaced now by two bands at 583 and 549  $m\mu$ , the second band being stronger and wider than the first. On making the solution slightly acid the four-banded spectrum of acid methaemoglobin type reappears. The two-banded absorption spectrum of the slightly alkaline solution was previously ascribed to a parahaematin compound, in other words to a compound between haematin and a nitrogenous substance or denatured protein. The fact that this compound easily reverts to the original acid state and that it is obtained when the solution is made only slightly alkaline suggests that the protein in this compound is not denatured and the compound therefore resembles alkaline methaemoglobin.

REDUCTION WITH  $\text{Na}_2\text{S}_2\text{O}_4$ 

A brownish red solution of peroxidase on addition of a reducer ( $\text{Na}_2\text{S}_2\text{O}_4$ ) turns bright red and its absorption spectrum is replaced by two bands a narrow and pale band  $\alpha$  ( $594.5 \text{ m}\mu$ ) and a very strong and very wide band  $\beta$  ( $558 \text{ m}\mu$ ) the two bands being united by a very faint shading. On oxidation the colour and the absorption spectrum of this solution revert to the original states.

The absorption spectrum of the reduced compound resembles somewhat that of reduced haemoglobin.

## COMPOUND WITH CO

On addition of carbon monoxide to the reduced compound its absorption spectrum is rapidly replaced by two very distinct bands  $\alpha$  ( $578 \text{ m}\mu$ ) and  $\beta$  ( $545.5 \text{ m}\mu$ ). This absorption spectrum resembles that of CO haemoglobin.

On passing a strong current of air through this solution which oxidizes the remaining  $\text{Na}_2\text{S}_2\text{O}_4$  and removes the CO present in the solution the compound reverts gradually to the original state showing the characteristic four banded absorption spectrum.

## COMPOUND WITH KCN

On addition of a small amount of KCN peroxidase solution turns deep red and its absorption spectrum is replaced by a very strong and wide band at  $542 \text{ m}\mu$  and a pale and narrow band at  $581.5 \text{ m}\mu$  with a shading between these two bands. The general appearance of the absorption spectrum conveys the impression of a single asymmetric band fading away towards the red end of the spectrum with a slight reinforcement at  $581.5 \text{ m}\mu$ .

This absorption spectrum resembles that of KCN methaemoglobin compound. It may be noted here that KCN is one of the most powerful inhibitors of peroxidase. In a concentration of  $0.001 \text{ M}$  it completely inhibits the activity of this enzyme.

## COMPOUND WITH FLUORIDE

NaF added to a slightly acidified peroxidase solution does not modify the type of its absorption spectrum but turns the solution green and changes the positions of the bands as well as their relative intensities. The absorption spectrum is still composed of four bands which now occupy the

following positions I, 615  $m\mu$ , II, 561  $m\mu$ , III, 529.5  $m\mu$  and IV, 496  $m\mu$ . The first band, as we see, is moved almost 300 A nearer the short wave end of the spectrum and it becomes much stronger than in the original preparation

The changes in the position and intensity of this band make it clearly visible in solid slices of horseradish root treated with fluoride. It is thus possible to estimate the concentration of this pigment *in situ* by comparing the intensity of this band with that of a known standard solution of purified peroxidase preparation also treated with sodium fluoride. NaF inhibits the activity of the enzyme. The inhibition is, however, not very marked. In concentrations of 0.003 and 0.001 M the inhibition is respectively 50 and 25 %

650	640	630	620	610	600	590	580	570	560	550	540	530	520	510	500	490										
	637	Acid methb				592				547.5					504											
		629.5	Catalase				580				544				506.5											
	645	Peroxidase					583				548					498										
$H_2O_2$ -peroxidase -I								561					539.5													
$H_2O_2$ -peroxidase -II							583				545.5															

FIG 2—Positions of the absorption bands of acid methaemoglobin, catalase, peroxidase and the two  $H_2O_2$  peroxidase compounds

#### COMPOUND WITH NO

The formation of a compound with NO can easily be followed in a Thunberg tube containing a peroxidase solution. The tube is evacuated, washed thoroughly with pure nitrogen and filled with pure NO. The four-banded absorption spectrum of peroxidase disappears and is replaced by two strong bands  $\alpha$  (570.5  $m\mu$ ) and  $\beta$  (539.5  $m\mu$ ). It is interesting to note that on opening the tube NO is rapidly oxidized to  $NO_2$  but the absorption spectrum of NO-peroxidase compound remains unchanged.

COMPOUND WITH  $H_2S$ 

The addition of  $H_2S$  to peroxidase solution replaces its absorption spectrum by two bands a faint band at  $587.5\text{ m}\mu$  and a strong band at  $549.5\text{ m}\mu$ . This compound is not affected by  $CO$ .

## COMPOUNDS WITH AZIDE AND HYDROXYLAMINE

$NaN_3$  and  $NH_2OH$  do not modify the absorption spectrum of peroxidase solution but they do modify some of the properties of peroxidase haematin which will be examined later on. We can mention here that  $0.003$  and  $0.001\text{ M}$   $NaN_3$  inhibit the peroxidase activity by  $75$  and  $40\%$  respectively while  $0.001\text{ M}$  hydroxylamine inhibits the enzyme activity by  $40\%$ .

## REACTION BETWEEN THE ENZYME AND SUBSTRATE

COMPOUND WITH  $H_2O_2$ 

On addition of  $H_2O_2$  to a strong solution of peroxidase the colour of the solution turns red and the three banded absorption spectrum is replaced by two bands which lie at  $561$  and  $530.5\text{ m}\mu$ .

The two banded absorption spectrum belongs to a compound of peroxidase with  $H_2O_2$ . This compound is however unstable and undergoes a more or less rapid decomposition liberating the peroxidase.

This decomposition of the  $H_2O_2$  peroxidase compound is mainly due to a substance present in peroxidase preparations which rapidly reacts with  $H_2O_2$  activated by the enzyme. The minimum amount of  $H_2O_2$  required for the formation of  $H_2O_2$  peroxidase compound can therefore be determined only when this substance is removed or its concentration greatly reduced. This can be attained either by purification of the enzyme preparation or oxidation of this substance by a gradual addition of  $H_2O_2$  until the two banded absorption spectrum of the  $H_2O_2$  peroxidase appears for the first time and then slowly disappears. The amount of  $H_2O_2$  subsequently added to bring back the absorption spectrum of  $H_2O_2$  peroxidase was found to correspond to one molecule of  $H_2O_2$  per atom of iron of total haematin present in the preparation.

On further addition of  $H_2O_2$  to a solution of enzyme which shows already a distinct absorption spectrum of  $H_2O_2$  peroxidase compound the absorption bands of the latter become diffuse and a new band begins to appear at  $583\text{ m}\mu$ . When the concentration of  $H_2O_2$  in solution reaches from  $15$  to  $25\text{ mol/g}$  atom of iron of peroxidase the absorption spectrum is completely changed and the two previous bands of  $H_2O_2$  peroxidase are replaced by

two very strong bands lying at 583 and 545.5  $m\mu$ . On further addition of  $H_2O_2$ , these two bands become stronger and they reach the maximum of intensity when the amount of  $H_2O_2$  added to the solution corresponds to about 100 mol. of peroxide per iron atom of peroxidase haematin.

During this change in the absorption spectrum the colour of this solution turns deeper red and  $H_2O_2$  undergoes rapid catalytic decomposition into water and molecular oxygen.

This new two-banded absorption spectrum belongs to a second compound formed between  $H_2O_2$  and peroxidase haematin. Owing to the rapid decomposition of  $H_2O_2$  when added in excess of the amount required for the formation of the first  $H_2O_2$ -peroxidase compound we have been unable to determine the exact amount of  $H_2O_2$  which is necessary for the formation of the second peroxidase-peroxide compound.

Peroxidase haematin forms therefore with  $H_2O_2$  two distinct compounds showing very different absorption spectra. The formation of the first compound probably requires one molecule of  $H_2O_2$  per atom of iron of peroxidase, while the formation of the second compound takes place only in presence of a great excess of  $H_2O_2$ . Both these compounds are unstable. The decomposition of the first compound is greatly accelerated by the presence in the solution of a substance reacting with  $H_2O_2$ . The decomposition of the second compound, which is not as rapid as that of the first compound, is due partly to catalytic decomposition of  $H_2O_2$  and partly to the destruction of the enzyme.

In purified enzyme preparations the decomposition of the first peroxide compound becomes much slower which makes possible a careful spectroscopic study of this compound. On addition of a little hydroquinone to such a stabilized  $H_2O_2$ -peroxidase compound the latter rapidly undergoes decomposition liberating the free peroxidase.

The same applies to the second peroxidase-peroxide compound. In other words, in presence of an acceptor or substance undergoing peroxidatic oxidation the  $H_2O_2$ -peroxidase compounds are rapidly decomposed. In these experiments hydroquinone can be replaced by other substances such as pyrogallol provided its concentration is so low that its oxidation product does not mask the colour of the enzyme. Hydroquinone can also be replaced by ascorbic acid or  $Na_2S_2O_4$ , which removes the  $H_2O_2$  and liberates the enzyme, but the reaction in this case is complicated by the ability of  $Na_2S_2O_4$  to reduce the peroxidase haematin.

The formation of the peroxidase-peroxide compound can be observed even in the first crude extract of horseradish of P Z 0.65 examined in a tube 6–10 cm. long. In this case, however, the addition of a large amount

of  $\text{H}_2\text{O}_2$  is required for the formation of the compound and only the absorption bands of the second peroxide compound which are much stronger than those of the first compound can be seen. The necessity for a great excess of  $\text{H}_2\text{O}_2$  in this reaction can be explained by the presence in this crude extract of a large amount of natural acceptor undergoing peroxidatic oxidation. In fact the addition of  $\text{H}_2\text{O}_2$  to this extract turns its colour distinctly reddish and this change in colour cannot be accounted for only by the formation of  $\text{H}_2\text{O}_2$  peroxidase compound as the concentration of the peroxidase haematin in this extract is much too small to affect the colour of the solution.

#### STATE OF HAEMATIN IRON IN $\text{H}_2\text{O}_2$ PEROXIDASE

In order to determine the valency of the iron in peroxidase haematin when it is combined with  $\text{H}_2\text{O}_2$  the following experiments have been carried out.

(1) 1 c.c. of a strong solution of peroxidase distinctly showing its absorption spectrum was put in each of three Ghunberg tubes and the appropriate amount of  $\text{H}_2\text{O}_2$  for the production of the first  $\text{H}_2\text{O}_2$  peroxidase compound was put in the hollow stoppers. The first tube as a control was left filled with air and of the remaining two one was filled with pure  $\text{N}_2$  the other with pure CO. The contents of the tubes were then mixed and rapidly examined with the spectroscope.

The absorption spectrum of the mixture in all three tubes was found to be that of the first  $\text{H}_2\text{O}_2$  peroxidase compound.

(2) In presence of a larger amount of  $\text{H}_2\text{O}_2$  but the same gases the absorption spectrum of each solution was that of the second peroxide peroxidase compound.

(3) Strong peroxidase solution distinctly showing its characteristic absorption spectrum was treated with  $\text{H}_2\text{O}_2$  while a rapid current of either  $\text{N}_2$  or CO was passing through the solution. When the first or the second compound with peroxide was obtained a drop of strong solution of hydroquinone was added to the mixture. The compound was rapidly decomposed liberating the peroxidase with trivalent iron which is shown by the appearance of the strong band at 645 m $\mu$ .

All these experiments show that in both compounds with peroxide the haematin iron of peroxidase remains in the trivalent state. In fact we have seen also that while the absorption spectrum of  $\text{H}_2\text{O}_2$  peroxidase compound is not affected by the addition of potassium ferricyanide when treated with  $\text{Na}_2\text{S}_2\text{O}_4$  its absorption spectrum is replaced by that of the reduced peroxidase haematin.



## EFFECT OF INHIBITORS ON PEROXIDASE HAEMATIN

Among inhibitors of the peroxidase reaction, KCN as we have previously seen forms with the peroxidase haematin a definite compound with a distinct absorption spectrum. On the other hand, sodium azide and hydroxylamine do not appreciably affect the absorption spectrum of peroxidase haematin.

However, if  $\text{H}_2\text{O}_2$  is added to a strong peroxidase solution in presence of sodium azide a new compound is formed with absorption bands lying at 590 and 550  $\text{m}\mu$ . This compound differs spectroscopically from both peroxide compounds previously described and resembles somewhat the reduced peroxidase haematin. That it is not a ferrous compound is shown by the fact that it does not react with CO and that only on addition of  $\text{Na}_2\text{S}_2\text{O}_4$  is a typical reduced peroxidase haematin compound formed.

As to hydroxylamine, the only evidence for its reacting with peroxidase haematin is that when added in excess it prevents the formation of a compound between peroxidase and  $\text{H}_2\text{O}_2$ .

It is important to note that  $\text{H}_2\text{O}_2$  has a great affinity for peroxidase haematin. Added in an equivalent amount to KCN peroxidase (in presence of 0.001 M KCN),  $\text{H}_2\text{O}_2$  replaces the KCN from peroxidase giving rise to the absorption spectrum of the first  $\text{H}_2\text{O}_2$  peroxidase. It is somewhat difficult to reconcile this observation with the fact that KCN strongly inhibits the peroxidase reaction tested in the usual way.

We must not forget, however, that the spectroscopic study of reactions between  $\text{H}_2\text{O}_2$ , KCN and peroxidase haematin and the study of the inhibitory effect of KCN on peroxidatic reaction are carried out under entirely different conditions. In fact, in presence of the same concentrations of  $\text{H}_2\text{O}_2$  and of KCN the concentration of peroxidase haematin in spectroscopic experiments is at least 200,000 times higher than the maximum concentration which can be used for estimation of peroxidatic activity. Moreover, while the former experiments are carried out in absence of substances undergoing oxidation or acceptors, the latter is carried on in presence of a large excess of an acceptor like pyrogallol.

## RELATIONSHIP OF THE CONCENTRATIONS OF TOTAL HAEMATIN AND PEROXIDASE HAEMATIN TO ENZYME ACTIVITY

It was shown previously that there is a definite proportionality between the concentration of total haematin and the catalytic activity of different peroxidase preparations. This proportionality was striking for all the

enzyme fractions from P Z 1 to P Z 400 in spite of different methods used in their preparation (Elliott and Keilin 1934). The activities of these preparations plotted against their haematin contents gave a series of points lying on a straight line. At P Z above 400 a distinct break in this line was noticed and a few points obtained with preparations between P Z 400 and P Z 818 were found to lie on another straight line forming a distinct angle with the previous one.

Our new preparations ranging from P Z 10 to 700 plotted in the same way gave a number of points lying on the first line described by Elliott and Keilin (1934). A very sudden break occurred however with a preparation of P Z 950-1000 the haematin content of which instead of being 1.68% dropped to 1.2%. The point given by this preparation was found to lie on the second line of Elliott and Keilin.

The fact that the break in proportionality may occur at two different levels of enzyme activity and depends on the methods of purification suggests that we are dealing here not with one haematin but with a mixture of haematin compounds which give the same pyridine haemochromogen. Peroxidase haematin represents therefore only a portion of the total haematin compound estimated as pyridine haemochromogen. We have found on the other hand that there is a strict proportionality between the catalytic activity of the enzyme preparations and the concentration of the unmodified haematin compound in these preparations measured by the relative intensities of the absorption band at  $645 m\mu$ . Estimated in this way the proportionality was found to hold good for all the enzyme preparations obtained so far which cover a wide range from P Z 10 to P Z 1500.

The relative concentrations of this haematin can be easily determined either by comparing directly the intensity of the band at  $645 m\mu$  or even better especially when dealing with preparations of lower activity by comparing the corresponding band of the fluoride derivative.

It is impossible however to determine the concentration of peroxidase haematin in terms of haemin or iron as we do not know yet whether the peroxidase haematin is the only haematin compound present even in our preparation of P Z 1500.

#### HAEMATIN COMPOUNDS IN INTACT ROOTS OF HORSERADISH

A slice of horseradish root 1 cm. thick cut out from a wide portion of the root and examined with a microspectroscope shows two absorption bands: a strong band at  $556 m\mu$  and a feeble band at  $645 m\mu$ . These two bands represent only portions of absorption spectra of two different

haematin compounds. The first band is the  $\alpha$  band of a haemochromogen which is very widely distributed in cells of plants and animals (Keilin 1925). The second (at  $645\text{ m}\mu$ ) is the band I of peroxidase. The  $\beta$  band of haemochromogen and the remaining bands of peroxidase, being masked by a general absorption due to other plant pigments, are invisible.

The two absorption bands which can be seen in the slice belong therefore to two bound haematin compounds: one (haemochromogen) with divalent iron, the other (peroxidase haematin) with trivalent iron. Having recognized these absorption spectra it is interesting now to determine the quantitative relationship between these two haematin compounds and the total haematin of horseradish, estimated as pyridine haemochromogen. These estimations can be carried out on the same slice.

(1) The haematin content of the natural *haemochromogen* was estimated in the usual way by matching the absorption spectrum of haemochromogen in the untreated slice with that of pyridine haemochromogen obtained from haemin. The concentration of the natural haemochromogen in terms of haemin was found to be from 0.00031 to 0.00046% of the wet weight of the roots. On treating a portion of this horseradish slice with  $\text{Na}_2\text{S}_2\text{O}_4$ , the absorption band of haemochromogen remains almost unchanged. In some places it increases slightly in intensity but it does not raise the total concentration of haemochromogen to a value higher than 0.00043–0.00051%.

(2) The concentration of peroxidase haematin in the slice can be only estimated approximately by transforming it into the fluoride compound (cf p. 123) and matching it with the fluoride compound of a purified peroxidase preparation, the haematin equivalent of which has previously been determined in the usual way. As on the other hand we are not certain yet that the peroxidase haematin even in our preparation of P.L. 1500 is the only haematin of this preparation, the value for the peroxidase haematin in the slice thus obtained will be, if anything, higher than its real value.

To transform the peroxidase haematin within the horseradish slice into its fluoride compound the slice 1 cm. thick is put into a Thunberg tube in a strong solution of NaF. The tube is evacuated to remove all the air from the slice and when the gas bubbles cease to appear the air is let in. The reaction is usually complete in 3–4 hr. The advantage in estimating peroxidase haematin as the fluoride compound is obvious: the band in the red becomes much stronger and is shifted towards the short wave side of the spectrum into the region where it can be much more easily seen. Estimated in this way it was found that the fluoride haematin band in a 1 cm. layer of horseradish was of the same intensity as the same band in a 0.375%

solution of peroxidase preparation of P Z 1000 examined in a layer 4 mm thick This shows that the P Z of horseradish should be at least 1.5

The total haematin of this purified preparation estimated as pyridine haemochromogen represents 1.2 % of its dry weight Assuming now that the whole of the haematin of this preparation belongs to peroxidase, the peroxidase haematin in horseradish slice would be approximately 0.0018 % As the total haematin of this slice estimated as pyridine haemochromogen is 0.003 %, not more and possibly much less than 60 % of it belongs to peroxidase haematin The remaining 40 % and possibly much more of the haematin which appears as pyridine haemochromogen is present partly as natural haemochromogen and partly in some other bound form

It may be noted here that in addition to the fluoride compound some other derivatives of peroxidase haematin can be seen directly in horseradish slices treated in an appropriate way Thus, the NO compound can be seen in a slice of root kept in a Thunberg tube, washed with  $N_2$  several times by evacuation and filled with pure NO

The peroxide compound can be obtained by soaking a number of slices 0.5 mm thick in  $H_2O_2$  and can be seen by examining spectroscopically a layer composed of 6-8 of these slices

#### SUMMARY AND CONCLUSIONS

(1) Strong horseradish peroxidase preparation in slightly acid solution is reddish brown in colour and shows an absorption spectrum of the methaemoglobin type composed of four bands I, 645  $m\mu$ , II, 583  $m\mu$ , III, 548  $m\mu$  and IV 498  $m\mu$

(2) On making the solution slightly alkaline (pH 10) the four banded absorption spectrum is replaced by two bands at 583 and 549  $m\mu$

(3) These two absorption spectra of peroxidase in acid and alkaline solutions belong to compounds with trivalent iron

(4) On addition of  $Na_2S_2O_4$ , the peroxidase haematin undergoes reduction its solution turns distinctly red and the four banded absorption spectrum is replaced by two bands at 594.5 and 558  $m\mu$  the first band being narrow and weak, the second wide and strong On the other hand, a boiled preparation of peroxidase when treated with  $Na_2S_2O_4$  gives the absorption spectrum of an ordinary haemochromogen

(5) The reduced peroxidase haematin forms a reversible compound with CO

(6) Oxidized peroxidase haematin combines reversibly with NaF, KCN,

$\text{H}_2\text{S}$  and  $\text{NO}$  forming compounds which show characteristic absorption spectra

(7) There is also evidence that it forms reversible compounds with sodium azide and hydroxylamine

(8) Peroxidase haematin reacts with  $\text{H}_2\text{O}_2$  forming two distinct compounds

(a) A compound formed in presence of a small amount of  $\text{H}_2\text{O}_2$  probably one molecule per atom of iron of peroxidase haematin and showing two absorption bands at 561 and 530  $\text{m}\mu$

(b) A compound formed in the presence of an excess of  $\text{H}_2\text{O}_2$  showing two very strong absorption bands at 583 and 545  $\text{m}\mu$

(9) Neither of these two compounds combines with  $\text{CO}$  and on addition of  $\text{Na}_2\text{S}_2\text{O}_4$  both compounds give a typical reduced peroxidase haematin. We have therefore no indication that the formation of these two compounds is accompanied by a change of valency of haematin iron

(10) These two peroxide compounds rapidly decompose liberating the peroxidase. The decomposition is much accelerated by the acceptor which is present in plant extract or by the addition of other acceptors such as ascorbic acid, hydroquinone or pyrogallol

(11) The  $\text{H}_2\text{O}_2$  peroxidase is thus an enzyme substrate compound, the formation and the reactions of which can be followed by direct spectroscopic examination

(12) There is no strict proportionality between the peroxidatic activity of the enzyme preparations and the concentration of total haematin of these preparations estimated as pyridine haemochromogen

(13) There is however a distinct proportionality between the enzyme activity (from  $PZ = 10$  to  $PZ = 1500$ ) and the concentration of the haematin compound estimated by the intensity of the absorption band at 645  $\text{m}\mu$  or other bands of different derivatives of this haematin, such as the fluoride compound. Peroxidase haematin in these preparations represents therefore only a portion of the total haematin estimated as pyridine haemochromogen

(14) The identity of this peroxidase haematin with the enzyme is strongly supported by the following evidence

(a) Proportionality between the enzyme activity and the concentration of the methaemoglobin like compound in preparations obtained by various methods

(b) Thermolability of this haematin compound similar to that of the enzyme

(c) Formation of compounds between this haematin and  $\text{H}_2\text{O}_2$

(d) Formation of compounds between this haematin and various substances which affect the catalytic activity of the enzyme

(e) The presence of a haematin compound in milk peroxidase preparations which has the same absorption spectrum the same properties and giving the same derivatives when treated by  $\text{Na}_2\text{S}_2\text{O}_4$  CO KCN NaF and  $\text{H}_2\text{O}_2$  as the peroxidase haematin from horseradish roots

(15) Peroxidase which shows great resemblance to methaemoglobin can therefore be considered as a compound of protohaematin with a native protein

(16) The same haematin nucleus combined with three different native proteins forms three distinct compounds methaemoglobin catalase and peroxidase which have many properties in common but show however striking differences in the nature and magnitude of their catalytic activities

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# A Rapid Method for Determining the Lowering of Tension of Exposed Water Surfaces, with some Observations on the Surface Tension of the Sea and of Inland Waters

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Ordinary methods of determining surface tension require a certain amount of apparatus and time and often disturb the surface thereby altering the tension of a water surface covered by a film of oil. As it was desired to make a number of observations on the amount of invisible contamination on the sea a method depending on the spreading power of drops of mixtures of different fatty substances has been worked out. An oil will just spread against an amount of contamination which lowers the tension by an amount equal to the spreading force, or 'spreading coefficient', of the oil.

Pure long chain hydrocarbon oils do not spread on clean water. If small amounts of substances containing water attracting groups in the molecule are dissolved in the hydrocarbon, spreading occurs, the spreading force depending on the amount of the second substance with the water attracting group in the solution. The theory has been treated particularly thoroughly by Langmuir (1933) this paper is however concerned only with the application of solutions of different concentrations of a substance such as dodecyl alcohol, in a pure, rather heavy hydrocarbon oil, to the determination of the surface tension of either fresh or salt water. It is found that the behaviour of a single drop of each of three or four such solutions, observed for half a minute or so, indicates the value of the surface tension within an accuracy of 1 dyne/cm. Calibration against water surfaces with oily contamination depressing the tension by known amounts is of course required, this is however quickly done once for all, and it is possible to find substances for the spreading solutions which behave similarly on waters differing widely in acidity and in salt content.

For the non spreading oil, a water white oil supplied by Shell Mex and

B.P., Ltd., K.A. 30, and stated to contain approximately 87 % of higher paraffins and 13 % of naphthenes, was used, for the spreading substance, *n*-dodecyl alcohol,  $C_{11}H_{23}CH_2OH$ ; the concentrations varied from 0.05 to 1.0 %. The spreading power of each solution was ascertained by placing a drop on a surface covered by a unimolecular film under known surface pressure (lowering of surface tension), in a trough similar to those commonly used for investigating these films on water (Adam 1926, 1930, pp 38 ff) In Table I are given the surface pressures against which the drops would spread slowly, to the nearest dyne/cm

TABLE I

% dodecyl alcohol	Surface pressure against which slow spreading occurs
0.05	Very slow spreading even on clean water
0.07	1
0.1	3
0.2	6
0.3	12
0.5	16
0.7	19
1.0	22

These figures were obtained on sea water. On distilled water values about 7 % lower were obtained, but this discrepancy may be just within the error of the experiment, and its cause has not yet been investigated. The surface tension of sea water is 1 or 2 dynes/cm higher than that of fresh, owing to the salts in it, but it is probable that these do not specifically affect the spreading force, which is equal to  $\gamma_W - \gamma_O - \gamma_{OW}$ , where  $\gamma_W$ ,  $\gamma_O$  are the tension of the water and oil respectively against air, and  $\gamma_{OW}$  the interfacial tension of water against the oil. The salts are likely to increase the surface tension of the water and the interfacial tension to the same extent. Small amounts of acid or alkali in the water made no difference.

Dodecyl alcohol was chosen instead of the more readily available hexadecyl (cetyl) alcohol, since the latter was insufficiently soluble in the hydrocarbon oil at room temperature. Substances such as oleic acid, which contain an acidic water-soluble group, are unsuitable, because small alterations in the acidity of the water make a great deal of difference to the interfacial tension while scarcely affecting the air-water tension, so that the spreading coefficient is very much altered. It was found that the small difference in acidity between distilled and sea water made a very great difference to the spreading of solutions of oleic acid.



The surface tension lowering (surface pressure) of any water surface can be estimated by placing drops of several of these solutions in turn on the surface, unless the surface is of large extent it is well to commence with the weaker solutions, otherwise if the stronger solutions spread they may temporarily contaminate the water surface. A drop will spread nearly as fast as on clean water, if the surface pressure is 3 dynes/cm lower than the figure for that concentration of alcohol given in Table I, against one 2 dynes higher it does not spread at all. There is little difficulty in noting the change in behaviour of a drop caused by a change of surface pressure of 1 dyne/cm, if the surface pressure is not over about 15 dynes/cm, when the accuracy is not quite so good. The solutions under 0.1 % always spread rather slowly, probably because the alcohol takes a little time to reach the interface between the water and oil.

It is possible to detect, and even roughly estimate the amount of, small quantities of oily contamination insufficient to lower the tension appreciably, i.e. quantities less than one complete unimolecular layer of oil. This is done by gently decreasing the area of the surface, collecting and pushing the contamination towards the drop of say 0.1 % dodecyl alcohol solution, which has been allowed to spread on the surface to a small patch generally showing interference colours, using a piece of wood or other "barrier" for collecting the contamination and moving it along the surface towards the drop. If there is appreciable contamination, the patch becomes flattened on the side nearest the approaching, generally quite invisible, contamination. The distance from the moving "barrier" at which the side of the spread patch becomes flattened is a rough measure of the fraction of a unimolecular layer originally present on the surface. The bows of a yacht moving in still water do this automatically, if there is a very little contamination, and the drop of 0.1 % solution is thrown just ahead of the bow and a little to one side, it spreads at first but becomes flattened on the side nearest to the yacht as it is overtaken. If the surface is really clean, the spread patch, which may have been formed about 2 yd. ahead of the part of the bow which approaches it, can reach the slight wave extending a few inches in front of the side of the yacht without appreciable distortion.

A set of a dozen or so small bottles, with thin glass rods in the corks, can be easily carried, mounted in a block of wood, and with their aid the determination of the surface tension of any open sheet of water becomes extremely simple.

The results of some measurements of tension by this method, taken in August and September 1936, on the sea near the shore, in harbours or creeks, and on inland waters, follow

Date (1936)	Place I Harbours	Lowering of tension	Remarks
Aug 24	Plymouth Sound, tide, half-ebb or lower.		
	In most places, including north side of Cattewater, middle of Sound generally, within a mile or so of Drake's Island, and main channel between Stone house and Cremyll	Less than 2	Probably clean
	But, within 2 ft of a muddy beach, half ebb, south side of Cattewater	6-20	
	Near shore, close to pier, small bay where many children were paddling on a hot day	10-20	
	Just to leeward of main sewage outfall	10	
	Close to west shore of Sound, up to 500 yd out, about half a mile length just north of Pickelcombe point	5-10	Faint <i>visible</i> traces of oil
Sept 16	Portsmouth Harbour tide, beginning of ebb *		
	Generally	Less than 2	
	But near Gosport jetty and in the oily looking track left by motor ferry boats	Over 20	
	Near Nicholson's yard	ca 15	
(N B The appearance of the ripples indicated an invisible film of oil roughly 5 yd wide along the Portsmouth shore, just below the dockyard )			
Aug 31	Milford Haven		
	(Close to shore) West Angle Bay (Milford Haven)	Less than 2	Probably clean
	Angle Bay (Milford Haven) (more confined than West Angle Bay)	2-3	
Sept 1	Pembroke Dock		
	Close to south shore	ca 5	
	Middle of channel	Less than 2	Probably clean
Sept 16	Stokes Bay *		
	Generally	Less than 1	
	In neighbourhood of Gosport sewage outflow	5-15	

\* I thank Mr H L Shute for the observations in Portsmouth Harbour and Stokes Bay.

Date (1936)	Place	Lowering of tension	Remarks
	<i>I Harbours—cont</i>		
	Open sea, close to shore (a few yards out only)		
Various	Thorpeness (Suffolk)	Less than 1	Probably clean
	Thurleston (South Devon)	"	"
	Newgale sands (Pembrokeshire)	"	"
	Swanbridge (small village near Cardiff)	"	"
	<i>But</i> , quite close to a very small sewage outfall at Swanbridge }	ca 10	
	Creeks and basins		
	A creek on Beaulieu river, several miles from mouth, 2 ft. from muddy shore }	ca. 15	
	Aldington basin, enclosed by lock gates (Shoreham harbour).		
	Leeward end	ca 18	
	Middle	ca 16	
	Windward end	ca 6	
	(The wind concentrates the invisible film to leeward )		
	Fawley creek (Southampton Water)	Less than 1	Probably clean
	Small basin, Fawley	1-2	
	Rivers and inland waters		
	Thames, at Putney Bridge (tide ebbing strongly)	Less than 2	
	Itchen, near mouth, at floating bridge between Southampton and Woolston }	Less than 2	
	Severn, at Aust-Chepstow ferry	"	
	Severn, at Newnham (end of ebb, water moving slowly down) }	About 10	
	Severn at Newnham (just after passage of "bore", very strong current flowing up) }	Less than 2	

N B All the above rivers are dirty looking, the Severn exceptionally muddy, yet all but one had practically clean surfaces.

## II. Norfolk Broads district

There was often some surface contamination, rather capriciously distributed. South Walsham Broad, and the Fleet Dyke close to it, showed little if any lowering of surface tension, Deep Dyke, at entrance to Hickling Broad, showed none at some times, up to 3 dynes at others (more in a visible patch of oil left by a motor boat). Horsey Mere showed parts clean and parts up to 7 dynes lowering, the Old Meadow Dyke between Horsey Mere and Heigham Sound, about 30-45 ft wide, was usually

clean in the middle, but the surface tension within 6 ft. of the sides was usually lower (lowering up to 10 dynes). The Bure, between Thurne mouth and St Benet's Abbey, had the tension 3-15 dynes low. These observations were not numerous enough to draw certain conclusions.

The main conclusions to be drawn seem to be as follows. Open-sea water, except near to sewage outfalls, muddy beaches, large numbers of people in the water, or visible patches of oil, is free from invisible films of oil. Creeks, small harbours, and confined surfaces of water may have their tension considerably below normal. Rivers flowing fairly fast generally have the normal surface tension, slower streams may have lowered tension. The tension is often lower near a bank than far from it. The transparency of the water is no guide to the cleanliness of its surface, in general moving water is cleaner than stagnant, on the surface.

#### SUMMARY

A very rapid method for finding the surface tension of natural water surfaces, based on observations of the behaviour of drops of solutions of a spreading oil in a non-spreading one, is described. Its accuracy is about 1 dyne/cm.

Preliminary results indicate that, in general, the surface tension of the sea near the coast is that of clean sea water, except very near to sewage outfalls or obvious sources of contamination. Creeks, small harbours, and confined areas of water may have their surface tension considerably below normal. The tension of river surfaces depends probably more on their speed of flow than on their transparency, some rapid, very muddy looking streams have the normal tension, while more sluggish, but clear streams or lakes may show considerable lowering of tension.

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## The Surface Membranes of Muscle Fibres

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### INTRODUCTION

The electrical properties of muscle are best explained in terms of the state of polarization of the surface of the muscle fibre. The uninjured surface of an isolated frog sartorius muscle is equipotential. Localized injury causes the injured part to be electrically negative to the uninjured surface. This indicates that normally the outside of the surface is positive to the inside. The same condition appears to exist in nerve fibres. Stimulation of muscle or nerve causes an impulse to move along the fibre. This impulse is a phase of depolarization which passes longitudinally along the surface of the fibre. The action potential which is a consequence of the depolarization has been much studied, but little is yet known of the nature and properties of the surface membrane at which the depolarization occurs. A consideration of the results of studies on the electrical behaviour of large single plant cells promises to throw some light on this problem (Osterhout 1929, 1931, 1934, 1935).

The protoplasm of a cell of *Valonia* or *Nitella* forms a layer about  $10\mu$  thick surrounding an aqueous vacuole. This protoplasm consists probably of an outer and inner layer of non-aqueous material and an intermediate aqueous layer. The evidence for this derives from (1) the shape of the action potential curve, and (2) the fact that a circuit consisting of cell sap—protoplasmic layer—cell sap has a considerable e.m.f., which would not be so if the protoplasm were homogeneous. It is the purpose of this paper to apply the method of the second criterion to muscle fibre, and to study this "asymmetry" potential. It is difficult to apply the same criteria to a muscle as to a large single algal cell for the following reasons. (1) A muscle comprises many fibres and the response of the inner fibres to a solute in

\* This work was done during the tenure of a Rockefeller Foundation Fellowship.

the external medium is governed by the time of diffusion of the solute into the muscle (2) An algal cell and a muscle fibre are histologically dissimilar (3) It is impossible to extract the semi liquid contents of a muscle fibre in the way that sap can be taken from a large plant vacuole And an aqueous solution cannot be prepared of the same inorganic salt composition as muscle fibre because the phosphate content is too high and causes precipitation of Ca and Mg

These difficulties are considered more fully below Instead of isolated fibres the whole sartorius of the frog was used A sartorius comprises 200-400 fibres running parallel each about  $40\mu$  in diameter (Fulton 1926 p 368) The longer fibres are 70-80 % of the length of the muscle i.e. about 2 cm in length The regular arrangement of fibres in the sartorius makes it likely that conclusions derived from the whole muscle are also valid for individual fibres when allowance is made for diffusion The gastrocnemius muscle is not suited to this kind of experiment because of the less regular arrangement of the fibres

This paper reports the results gained by using crushed muscle instead of sap and aqueous solution approximating in composition to the inorganic salt content of the muscle interior

The effects of these agents on the intact muscle are discussed in the light of the electrical properties of the surface membranes of the muscle fibres

#### THE STRUCTURE AND COMPOSITION OF MUSCLE FIBRES

The individual muscle fibre consists of a viscous interior enclosed in a tougher elastic external membrane The interior although semi liquid has a definite structure which is revealed by staining or by examination of the living fibre with ordinary light polarized light and with X rays Further evidence on the nature of the fibre interior is derived from physico chemical studies Hill and Kupalov (1930) from a survey of the analysis of muscle and from experiments on its vapour pressure decided that all the ions present in muscle are present in free solution in the free water of the muscle No ions are immobilized or bound A difficulty in this conclusion is that the concentration of calcium magnesium pyrophosphate and phosphate ions in muscle far exceeds the values corresponding with saturated aqueous solutions of calcium and magnesium phosphates and pyrophosphates This may be because the pyrophosphate and phosphate are present as part of large organic molecules which prevent them acting as precipitants for Ca and Mg ions But if this is so it is difficult to understand how they are free to exert an osmotic pressure

Comparing the muscle fibre with *Valonia* or *Nitella*, the external membrane of the fibre corresponds with the protoplasmic membrane of the plant cell and the fibre interior with the vacuole. The analogy is not close because the fibre interior is contractile, viscous and rich in protein, but it resembles the aqueous solution present in the vacuole of *Nitella* or *Valonia* in that the inorganic content of muscle exists as ions in the "free" water of the muscle.

#### COMPOSITION OF MUSCLE BREI AND OF THE EXPERIMENTAL SOLUTIONS

The original desire to apply the contents of the muscle fibre to its outer surface is difficult to realize. Instead of this the whole muscle was crushed and the brei so obtained used. This introduces several errors. The contents of the interfibrillary spaces and the material of the surface membrane are included with the contents of the fibres, and all these differ in composition. Also the act of crushing initiates chemical changes which make the brei differ still further from the interior of the resting muscle. These changes resemble those caused by extreme fatigue of the intact muscle, but have been studied less. They have to be taken into account in making up the substitute solution. They are an increase in lactate, phosphate, ammonium, and hydrogen-ion concentrations, and in creatine (lactic acid, Fletcher and Hopkins 1906, ammonia, Parnas and Mozolowski 1927, creatine, Needham 1931, p. 68, Hill and Kupalov 1930, p. 469). The increases are greater still in death rigor than in fatigue, and presumably resemble those caused by crushing. The effect of crushing the muscle was investigated experimentally in two particulars, the vapour pressure and the pH.

Resting muscle is isotonic with 0.725 % NaCl solution. Fatigue causes an osmotic increase of 0.35 % and rigor of 0.45 %. Muscle in rigor is thus isotonic with 1.175 % NaCl solution. There seem to be no direct observations on the vapour pressure of muscle brei. It has presumably the same approximate value. To check this the vapour pressure of muscle brei was determined by direct comparisons with solutions of NaCl by Barger's method. It was found that the osmotic pressure of fresh brei is the same as that of about 1.06 % NaCl (cf. fatigue 1.075 %, rigor 1.175 %) but increases slowly with time.

The pH of resting muscle fibre is about 7 and of fatigued muscle about 6.5 (see Hill and Kupalov 1930). These figures rest on measurements with the glass electrode on muscle extract, on colorimetric observations with phenol-red injections, and on calculations from the Henderson-Hasselbalch equation, knowing the  $\text{CO}_2$  pressure. Meyerhof (1930, p. 21) found pH 7.3 for resting muscle, pH 6.5 for fatigued muscle, and pH 6 for muscle killed with chloro-

form. He used the quinhydrone electrode with an aqueous muscle extract. In the present experiments the quinhydrone electrode was used directly with the crushed muscle. This electrode has been used satisfactorily for pH measurements on the muscles (Benson 1928) and nerves (Root 1934) of fish. Clark (1928) refers to the protein error of the quinhydrone electrode. This is apparently serious with haemoglobin but is not general since with gelatin solution and gels up to 3 % the electrode is trustworthy (Francis 1931) as well as with muscle and nerve which contain much protein. The value obtained with fresh brei was  $pH\ 7.05 \pm 0.1$ . On standing the pH fell to a steady value of  $pH\ 6.4 \pm 0.1$  reached after about 18 hr.

Using all available data the following composition was finally adopted for the aqueous solution resembling brei (given in molalities)

## Solution A

K <sup>+</sup>	0.109	Cl	0.116	Total molality 0.39 i.e. equivalent to 1.14 % NaCl
Na <sup>+</sup>	0.038	PO <sub>4</sub>	0.010	
Ca <sup>++</sup>	0.007	HCO <sub>3</sub>	0.007	
Mg <sup>++</sup>	0.014	Lactate	0.053	
NH <sub>4</sub> <sup>+</sup>	0.005	Creatine	0.023	

pH 6.6

The phosphate figure cannot be increased to the actual value for muscle because it already represents the limit of solubility. Chloride is used instead.

The other experimental solutions were made by the appropriate mixing of the following solutions: NaCl 0.124 M, KCl 0.124 M, CaCl<sub>2</sub> 0.086 M and solutions of primary and secondary phosphates of potassium and sodium of pH 7 and 0.116 M with respect to phosphate. These solutions are all isotonic with resting muscle, i.e. hypotonic to the brei. Analyses have shown that the interior of the muscle fibre is rich in potassium and phosphate and the spaces between the fibres rich in sodium and chloride (Gellhorn 1929, p. 103; Mond and Netter 1930, 1931). Solution 4 (Table II) containing 0.116 M potassium phosphate buffer pH 7 thus represents the nearest approach that can be made with inorganic solutions to the fibre contents. Solution A 1 represents the nearest possible approach with available substances to reproduce the inorganic and organic content of the whole muscle. It contains

Creatine 0.027 M, adenosine (from yeast) 0.007 M, adenylic acid (from yeast) 0.008 M, orthophosphate 0.01 M, lactate 0.003 M, bicarbonate 0.007 M and the remainder chloride and the cations in the proportion in which they exist in resting muscle.



## DIFFUSION POTENTIALS

It was of interest in the interpretation of later results to know the diffusion potentials ( $E_{ab}$ ) between brei and the various experimental solutions used. The circuit measured consisted of

Calomel electrode	Saturated KCl	Thread im- mersed with solution	Fresh brei	Saturated KCl	Calomel electrode
		$P_1$	$P_2$	$P_3$	

The figures obtained are shown in Table II

If we assume that the potentials at the interfaces saturated KCl brei ( $P_3$ ) and saturated KCl solution ( $P_1$ ) cancel out, the figures in the table measure the potential  $P_2 = E_{ab}$  at the brei solution interface. The electrolytes in the solution tend to diffuse into the brei and the electrolytes in the brei tend to diffuse out into the solution. Between solutions 3 and 4 a series of solutions was used in which the proportion of Ca and Cl was progressively diminished and those of K and  $\text{PO}_4$  increased. As this is done the value of  $E_{ab}$  falls and finally (solution 4) is reversed in sign. The sign of  $E_{ab}$  shows (for solutions 1, 2, and 3) that the tendency of cations to diffuse faster than the anions is greater in the direction brei to solution than in the reverse direction. That this is indeed the case for the phosphate containing compounds in muscle is shown by the figures of Rothschild (1929) obtained from dialysis experiments. This tendency is progressively diminished and finally reversed by changing the composition of the solution so that the proportions of fast-moving cations (K) or slow-moving anions ( $\text{PO}_4$ ) are increased. Except in buffered solutions the potential changes slowly with time in the sense demanded by the increasing acidity and electrolyte concentrations of the brei with time.

## EXPERIMENTAL

Sartori of small frogs (*Rana pipiens*) were used almost exclusively 3-4 cm in length and 1-2 mm in thickness were usual dimensions. The sartori with the knees attached were dissected, separated at the pubis with a minimum of injury and left in Ringer solution for 1 hr. After this treatment no potential difference above 1 mV existed over the intact muscle surface. Comparisons of the effect of brei and experimental solutions were made by the simultaneous use of twin sartori. Each intact muscle was carefully blotted and placed with the ends in separate pits (2-3 c.c. capacity) in a block of paraffin wax. The part of the muscle exposed between the pits was placed upon, and covered with, a layer of vaseline. One pit was filled with

Ringer solution and the other with the experimental solution or with brei. The whole preparation was kept under a glass cover between measurements. In this way short circuits and evaporation were avoided and the treatment could be studied for long periods. Connexion to the calomel electrodes was made from the brei with a thread moist with saturated KCl solution and from the experimental solutions with threads soaked in the particular solution used. The errors due to short circuits along the uninjured or untreated surface of the muscle must be guarded against. When injury potentials are being measured the best way to make contact with a cut end of the muscle is by means of filter paper or thread soaked in the solution.

A constant short circuit is made by the liquids between the fibres. Hober (1905) showed that preliminary soaking of the muscle in sugar solution which washes out the electrolytes between the fibres increases the value of the injury potential. Experiments in confirmation of this showed that if twin sartorii are soaked for 2 hr. one in Ringer and the other in isotonic sucrose solution the injury potential in the latter case is 20–25 % greater than in the former but does not survive as well. Sugar treatment also abolishes the irritability and enhances the oxygen consumption of muscle (Fenn 1930). Because of these signs of harm to the muscle soaking in sucrose solution was avoided as a routine except when especially desired.

#### EFFECT OF MUSCLE BREI ON INTACT MUSCLE

The effect of brei applied to the intact muscle was in the same sense as the effect of cutting. The place treated became negative in the external circuit to the rest of the intact surface. The potential increased at first just as the injury potential of cutting does. After about 1 hr. it stayed steady. Twin sartorii gave almost identical results if the brei was applied to the thigh end of one and the knee end of the other. When the brei had produced a steady potential (*A* fig. 1) the muscle was cut at the brei treated part and the cut surface left in contact with the brei. The potential rose at once to a new steady value *B*. This is the injury potential of the muscle when brei is in contact with the cut surface or in other words the resting potential of the uninjured muscle surface measured on the following system

+Calomel	Sat	Ringer	Intact	Muscle	Cut brei	Sat	Calomel
	KCl	solution	surface	interior		KCl	

The sign refers to the potential as measured in the external circuit. The steady potential previous to cutting is that of the system

+Calomel	Sat	Ringer	Intact	Muscle	Intact	Br i	Sat	Calomel
	KCl	solution	surface	interior	surface		KCl	

The abrupt rise (*A* to *B*, 7 mV) on cutting is the difference of these systems and therefore measures the p.d. of the chain

-Muscle interior | Intact surface | Brei<sup>+</sup>

Table I indicates the corresponding potentials for the protoplasmic membranes of some large algal cells when the following system is measured

Sap in vacuole | Protoplasmic membrane | Sap outside

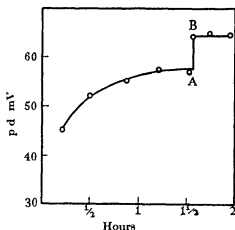


FIG. 1.—Effect of crushed muscle applied to the exterior of an intact sartorius. Equilibrium potential at *A*. On cutting muscle at treated part injury potential *B* is obtained.

TABLE I

Material	Resting potential mV	Asymmetry potential mV
Muscle	+ 57	+ 7 (brei)
<i>Nitella</i>	+ 200	- 15
<i>Valonia</i>	- 10	- 65
<i>Halcyotis</i>	+ 70	+ 50

The sign is that of the external surface in the external circuit. The figures for muscle are the average of 33 separate determinations.

Assuming that the seat of the potential in muscle is at the surface membrane of the fibre and that brei is the same as the fibre contents, 7 mV is a measure of the electrical asymmetry of the surface membrane. This is of the same sign as the asymmetry of the protoplasmic membrane of *Halcyotis* and opposite in sign to that of *Nitella* and *Valonia*. The asymmetry in the algal cells is probably due to the difference in composition between the outer and inner non-aqueous layers of the protoplasmic membrane.

The same explanation may hold for the surface membrane of the muscle fibre or, more simply, the results indicate that the internal and external surfaces of the muscle-fibre membrane are electrically different

The effect of brei on the resting potential of intact muscle is reversible. Long treatment with brei (2 hr or more), however, destroys the complete reversibility and also injures the muscle which becomes swollen and opaque.

#### EFFECT OF EXPERIMENTAL SOLUTIONS

The various solutions prepared as described above were used in exactly the same way as the brei. Their effects on the muscle potential were the same in respect to reversibility and time relations as the effects of brei. The potentials developed are shown in Table II. In each experiment twin sartori were compared, one being treated with brei and the other with the experimental solution. Each figure represents the mean of at least four separate experiments.

TABLE II

Solution	Diffusion potential against brei $E_{s,b}$	Difference between solution and brei effect $E_s$	Rise in potential on cutting at steady state $E_{c,s}$	$E_d$	$E_i$
			$E_{c,s}$		
1. Ringer solution pH 7	16	55	48	—	—
2. Solution A	12	14.5	7.5	48	27
3. KCl 0.109 M, $\text{CaCl}_2$ 0.01 M	9.5	14	6	47	30.5
4. Potassium phosphate buffer 0.116 M pH 7	-4.5	-5	0	32	39
5. Solution A1	-2	-2	0.5	39	40

It is seen that the values of  $E_{s,b}$  and  $E_c$  decrease side by side. The value of  $E_{c,s}$  however remains between 5 and 7.5 mV for all solutions containing 0.019 M of potassium ion (the concentration present in muscle) irrespective of the value of  $E_{s,b}$ . Only when the concentration of potassium exceeds this value, e.g. in nos. 4 and 5, does the value of  $E_{c,s}$  fall.

The rise in potential on cutting at the steady state,  $E_{c,s}$ , is a measure of the difference between the two following systems

Muscle interior		Membrane		Solution (before cut)
$E_1$				$E_s$
and				
Muscle interior		Solution (after cut)		
		$E_s$		

Thus for solution A,  $E_{c,s} = (E_1 + E_s) - E_s = 7.5$ . But, assuming muscle

interior and brei are the same in respect of diffusion potentials, it is known that  $E_s = E_b = 12$ , therefore  $E_1 + E_2 = 19$  mV. Since solution A approximates in inorganic content to the muscle interior this potential indicates considerable electrical asymmetry of the surface membrane of the muscle fibre. The asymmetry revealed by the use of brei amounted to 7 mV, a much smaller value. This discrepancy suggested that diffusion potentials may play a part in the effect of solutions on the muscle surface. This suggestion is supported by the fact that the difference between the solution and the brei effect ( $E_c$ ) varies in the same sense as the diffusion potential between the solution and brei. This indicates that the potential  $E_1 + E_2$  includes a diffusion potential which is of the same order as that of the solution against brei. This diffusion potential which exists between the solution and the intact surface or between the solution and the fibre interior *across* the intact surface, was investigated further as follows.

#### DIFFUSION POTENTIALS AT THE MUSCLE SURFACE MEMBRANE

Two series of solutions were used. One consisted of the solutions in Table II and the other of the same solutions diluted one-tenth with isotonic sugar solution. The method of mounting the muscles was the same as before. Two intact sartorii were equilibrated for 1 hr. in Ringer solution and then left in isotonic sucrose solution for 1 hr. with several rinsings. This should remove the liquids between the fibres. One sartorius is cut and brei applied to the cut end and the injury potential measured with the sugar-diluted solution on the intact surface. This gives the p.d. of the system

Sat KCl	Solution C/10	Intact surface	Muscle interior	Cut brei	Sat KCl
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This is the potential ( $E_d$ ) across the single intact surface with adventitious diffusion potentials so far as possible minimized. The other twin muscle is kept intact and treated with the diluted solution at one end and the plain solution at the other. The system then measured is

Sat KCl	Solution C/10	Intact surface	Muscle interior	Intact surface	Solution C	Sat KCl
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This potential ( $E_s$ ) is the algebraic sum of the potentials across the surface membrane at the point of application of the two different solutions. The values of  $E_d$  and  $E_s$  for the different solutions are given in Table II. Again, it is seen that the order of the effect of the solutions on the intact surface is the same as the order of the diffusion potentials of the solutions against brei.

## THE ROLE OF DIFFUSION

Before proceeding to the discussion of the foregoing results it is necessary to compare the observed times taken for steady potentials to develop with the brei or solutions, with the times calculated for the muscle to come to diffusion equilibrium with substances in solution. For the purposes of calculations a sartorius may be treated as a solid bound by two parallel surfaces exposed to the diffusing substance at each surface. For this case an equation has been given by Hill (1928)

The only substances apart from carbon dioxide and oxygen for which the diffusion constants in muscle are known are lactic acid and sodium phosphate at pH 7.2 (Eggleton, Eggleton and Hill 1928, Stella 1928). The diffusion constants of the substances present in brei and in the solutions used are probably of the order of  $10^{-5}$  in resting muscle. The comparison shown in Table III may then be made

TABLE III

Substance	Diffusion constant	Equilibrium time for muscles 1-2 mm thick (min)	
		Calculated	Found
	$10^{-4}$	25-100	For muscles treated with brei or experimental solutions
	$10^{-5}$	250-1000	
Lactic acid (resting muscle)	$6 \times 10^{-5}$	167-668	30-90
Lactic acid (fatigued muscle)	$5 \times 10^{-6}$	500-2000	—
Sodium phosphate (fatigued muscle)	$5 \times 10^{-6}$	500-2000	—

The difference between the times calculated for saturation by diffusion and the times experimentally found for maximum potential to develop is considerable. This means that penetration of the diffusing substance into the interior of the muscle fibre is far from complete by the time the diffusing substance has produced its maximum effect on the resting potential. Now it is likely that diffusion into the spaces between the fibres occurs at the same speed as in aqueous solution, i.e. 10-100 times faster than through the whole muscle. On this assumption (i.e.  $K = 10^{-4}$ ) the time calculated for the spaces between the fibres to come to equilibrium with a diffusing substance is of the same order as the time taken for the resting potential to reach a new steady value. Once the spaces between the fibres are filled with diffusing substance the latter has immediate access to the fibre surfaces which are the seat of the resting potential. In this way it is possible

for the diffusing substance to achieve its effect on the resting potential long before it has penetrated the interior of the fibres to saturation point.

### DISCUSSION

The conclusions that may be drawn from the measurements described in this paper are best made clear with the aid of a diagram (fig 2). A single fibre, representing one of the units of which the sartorius is built up, is depicted cut at *C*. The external membrane is shown with an inner and outer

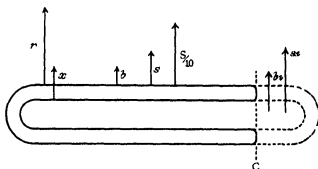


FIG. 2.—Diagram of hypothetical single potentials at the surface membrane of a single muscle fibre. See text for key to symbols.

surface. The potential between the inner surface and the interior of the fibre is indicated by the arrow  $x$ . The potentials existing between the outer surface and Ringer solution, brei, experimental solution and the same diluted to one-tenth are indicated by arrows  $r$ ,  $b$ ,  $s$ , and  $S/10$  respectively. The arrows  $as$  and  $bs$  indicate the potentials between the experimental solution and brei, and the fibre interior. These single potentials have no real meaning but are a useful mental aid. It is their differences that are experimentally measured.

The potential caused by brei treatment ( $E_b$ ) is thus, in terms of the separate potentials figured,

$$E_b = r + x - (b + x) = r - b = 57 \quad (1)$$

When the muscle is cut the potential becomes

$$E_c = r + x - b_s = 64 \quad (2)$$

Whence, by subtraction,

$$x + b - b_s = 7 \quad (3)$$

Hitherto it has been assumed for simplicity that  $b_s = 0$

By similar arguments the potentials found experimentally (Table II) can be expressed in terms of the single potentials presumed to exist. Thus

$$E_{cs} = s + x - s_1 \quad (4)$$

$$E_s = s/10 - s \quad (5)$$

$$E_d = s/10 + x - b_1 \quad (6)$$

$$E_d - E_s = s + x - b_1 \quad (7)$$

Using these equations one can evaluate the asymmetry potential (a p) of the surface membrane of the muscle fibres. This term should strictly speaking only be used to designate the potential across the surface membrane when the fibre contents are present on both sides. It is used here however for the potentials obtained when brei or various substitute solutions are used on the outside surface. The a p obtained with brei is +7 mV (equation (3)). This assumes the potential ( $b_1$ ) between brei and fibre interior is zero. Actually it is probably small and positive. Ascribing it to the difference in potassium concentration difference between brei and interior (0.109 and 0.124 M respectively) it would be +3 mV thereby making the a p for brei +10 mV reckoned outwards from the fibre surface.

Two sets of values for the a p with solutions are obtained: one using the same method as with brei (equation (4))

$$\text{a p} = s + x = E_{cs} + s_1$$

and the other using solutions diluted with sucrose (equation (7))

$$\text{a p} = E_d - E_s = s + x - b_1$$

The a p's by these two methods are compared in Table IV. In deriving these values it is assumed that  $b_1$  is zero and that  $s_1 = E_{sb}$ , that is that the diffusion potential between solution and fibre interior is the same as between solution and brei. The error in these assumptions is likely to be small and in the same direction in each column. This is borne out by the very fair agreement of the values for the a p's obtained by the two different methods. It is clear that the a p varies with the diffusion potential of the solution against brei. Further measurements not recorded here confirm this for solutions intermediate in composition between nos. 3 and 4. This indicates that the surface membrane of the fibre is the seat of a diffusion potential and that the ionic mobilities have the same relative order through the membrane as in free solution. It may be seen from the equations that  $x$  the potential difference presumed at the interior between fibre interior and



membrane, cannot be separated from the other potentials. It must be considered together with the potential at the outer surface of the membrane as part of the diffusion potential between the inside and outside of the fibre.

TABLE IV

Solution no	Diffusion potential against brei $E_{db}$	Asymmetry potential		Asymmetry potential mean value
		$a p = s + x - b i$ $= E_d - E_i$	$a p = s + x$ $= E_{ds} + s i$	
5 (A 1)	-2	-1	0.5	0
4	-4.5	-7	-4.5	-5.5
3	9.5	16.5	15.5	18
2	12	21	19.5	20

The solutions most nearly resembling the fibre interior which have so far been used are isotonic potassium phosphate buffer (no. 4) and solution A 1 (no. 5). These give a  $p$ 's reckoned inwards of 5.5 and 0 mV respectively. It has not proved feasible in making these substitute solutions to use the phosphorus-containing substances in the form in which they exist in muscle. Such compounds, adenine pyrophosphate, hexose phosphates, and creatine phosphoric acid, by giving large anions of low mobilities (Rothschild 1929), would give a greater  $a p$  reckoned inwards. Similarly, the use of the muscle proteins at pH 7, which is on the alkaline side of their isoelectric points, by providing large anions would again tend to increase the  $a p$  reckoning inwards. If these improvements could be made a final value of 10–20 mV for the  $a p$  directed inwards may be expected (compare Table I). It should be pointed out that in all this work complete osmotic freedom of the ions within the fibre is assumed. If a proportion of the ions is bound then the ionic concentration of the solution necessary to resemble the fibre interior will be less and as experiments show the  $a p$  will be correspondingly greater.

#### THE POTASSIUM EFFECT

Previous workers have shown that when solutions of potassium chloride of various concentrations are put on the muscle, the resting potential is related logarithmically to the potassium concentration used

$$E = E_0 + \frac{RT}{F} \log \frac{K}{K_0},$$

where  $K$  is the potassium concentration and  $E_0$ ,  $K_0$  are constants. (Hober 1926, p. 732, Hegnauer and others 1934.) This has been taken to mean that the surface membrane of the fibre is permeable only to potassium ion, the concentration of which thus controls the potential. Recent stoichiometric

work (Fenn and Cobb 1934 Mond and Netter 1931) shows however that permeability to other ions e.g.  $H$  and  $HCO_3$  also exists and Hober (1926) and Seo (1924) have shown that the resting potential varies with different ions according to their position in the Hofmeister series. This however is related to the effect of the ions on the colloidal stability of the fibre surface rather than to the mobilities of the ions through the surface. The potassium ion is thus not unique though it is paramount where the permeability and potential across the fibre surface are concerned.

The potentials  $E_d$  ranging from 32 to 48 mV between solutions differing in  $K$  concentration by a factor of 10 should all be equal to 58 mV if the muscle surface were permeable only to  $K$  ion. Similarly the injury potential of a muscle in Ringer solution should be 104 mV if only the  $K$  concentration difference between inside and outside were concerned. Actually it is 64 mV. The difference is too great to be due to short circuiting by the liquids between the fibres which at the most causes a loss of 20 % of the potential and this loss was avoided in the measurements of  $F_d$  by preliminary soaking in sucrose solution. Clearly the potassium ion alone does not control the potential differences observed. The work reported in this paper draws attention to the contribution made to the potential across the surface membrane of the fibre by other ions when the potassium concentration is kept constant. All the measurements (Table II) show that the potentials are symbatic with the diffusion potentials of the solutions against brine that is with the relative mobilities of the anions and cations in aqueous solution and also presumably through the membrane.

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#### SUMMARY

Experiments on the nature of the electrical potential existing across the surface membranes of the fibres of frog muscle are described. Solutions approximating in content to the composition of the interior of the muscle fibre were prepared. These were applied to the outside of the muscle and the potentials set up were measured. From the measurements it was concluded that if the contents of the muscle fibre were applied to the outside a potential across the membrane of 10–20 mV directed inwards would exist. This asymmetry potential points to a difference in electrical properties between the inner and outer surface of the membranes of the muscle fibres.

The effects of the various ions present in muscle on the surface membrane potential were also studied and are discussed. Apart from potassium it was found that the effects of the ions could be related to their mobilities in aqueous solution.

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## The Relations between Surface $pH$ , Ion Concentrations and Interfacial Tension

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The  $pH$  of a surface phase cannot be estimated by the methods available for bulk phases in this paper an attempt is made to estimate the  $pH$  of a surface phase by analysing the behaviour of carboxyl groups in an adsorbed interfacial film. The work of Donnan (1899), Jahrisch (1922), Hartridge and Peters (1922) and Peters (1931) has shown that the interfacial tension between solutions of long chain acids or amines and aqueous solutions is much lowered when the  $pH$  of the aqueous phases is such that the end group of the acid or amine which forms the interfacial film between the immiscible solvents is electrolytically dissociated. The interfacial tension is low on the alkaline side and high on the acid side for the fatty acids but low on the acid side and high on the alkaline for the amines. The change of tension takes place over the  $pH$  range 5–10 and the form of the curves relating interfacial tension to  $pH$  is strongly reminiscent of the change of dissociation with  $pH$  indicating that there is a close relationship between the interfacial tension and the degree of dissociation.

Close inspection of Peters's curves shows however that the range of  $pH$  over which the  $\gamma$  change occurs with the fatty acids is about 3 units of  $pH$  more alkaline than the range over which dissociation occurs in bulk phases also that dilution of the buffer solutions without change in  $pH$  may cause a rise in  $\gamma$ . Accepting the view that the changes in  $\gamma$  are due largely to changes in ionization of the carboxyl or amino groups in the surface film these results strongly suggest that the  $pH$  in the surface layer may not be the same as in the bulk aqueous phase.

Such differences of  $pH$  between surface and bulk phases in equilibrium with one another might be of great importance in biological processes. In this work further experimental evidence is obtained of this difference and it is shown theoretically that it may amount to several units of  $pH$ . The

\* The abbreviation  $\gamma$  will be used for interfacial tension.

influence of the specific nature of the cations in the aqueous phases is also investigated. The interfaces investigated were partly chosen for their suitability for a later investigation of the behaviour of protein films at interfaces.

#### EXPERIMENTAL

Oleic and palmitic acids, cholesterol, and lecithin were used, dissolved in brombenzene. The brombenzene was purified, usually by shaking with solid barium hydroxide, followed by fractional distillation under reduced pressure, until there was no diminution in the interfacial tension against distilled water after standing in contact for 24 hours. Brombenzene is an excellent solvent for the materials used, is easily purified from surface-active impurities, has a low vapour pressure, and is denser than water. The last property is particularly convenient for tensimeter measurements.

The palmitic acid (Eastman Kodak Co.) was recrystallized twice from methyl alcohol and three times from benzene. The cholesterol initially contained an acid impurity which was not removed by three crystallizations from benzene, but five crystallizations from methyl alcohol, followed by three from benzene, were sufficient to abolish nearly all the lowering of  $\pi$  by alkaline solutions. The oleic acid ordinarily used was the best supplied by the Eastman Kodak Co., a part of this was purified by the method of Lawrence (1929) without materially affecting the results. Lecithin was purified by the method of Levene and Rolf (1927).

All solvents, including water, were distilled from an all-glass (Pyrex) apparatus. The buffer solutions were acetate, borate, phosphate, and glycine, made up according to Clark's directions but with the strengths adjusted to 0.4 M in cations, not anions, as is the usual procedure. To avoid surface-active impurities in the soda, clean metallic sodium was dissolved in water covered by ether, as described by Clark (1928). pH determinations were made with a glass electrode (cf. McInnes and Belcher, 1933), using constant boiling hydrochloric acid as standard.

Most of the  $\pi$  measurements were made with du Nouy's tensimeter (1925), checked occasionally by drop-weight and capillary rise measurements. The drop-weight results were usually a few tenths of a dyne higher than the tensimeter, and the capillary rise, a similar amount lower, possibly because the contact angle was not zero. Measurements were made at  $21^\circ \text{C} \pm 1$ .

All apparatus was cleaned with hot chromic acid, followed by soaking in clean tap water for 24 hours, or, with vessels required for a long series of measurements, by steaming.

# RESULTS

## *a—Effect of Concentration of Organic Solute, in Absence of Salts*

Fig 1 shows that the maximum, final lowering of  $\gamma$  varies a great deal with different solutes, but that it is reached practically in all cases when the bulk concentration is 1%. The values given are final ones, obtained

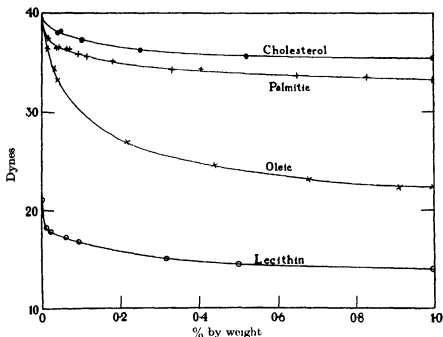


FIG 1—Interfacial tension and concentration of surface-active substances ● Cholesterol, + palmitic acid, x oleic acid, ○ lecithin

after the  $\gamma$  had become steady, which took some hours with the more dilute solutions, at 1%, steady values were obtained in not more than 10 min. The limiting values for the lowering of  $\gamma$  are given in Table I.

TABLE I—REDUCTION OF  $\gamma$  BROMBENZENE AGAINST DISTILLED WATER BY ADDITION OF 1% OF VARIOUS SUBSTANCES

	Dynes
Cholesterol	4.1
Palmitic acid	6.3
Oleic acid	17.5
Lecithin	25.5

The reproducibility of individual measurements was usually 0.2 dyne, except for the cholesterol solutions, when it was about 0.5 dyne, the points are the mean of two or more determinations

*b—The Effect of Variation of pH and Cation Concentration*

It was desired to work mainly with surfaces on which the maximum amount had been adsorbed, so that the surface concentration of dissociating groups should not vary. As the experiments of the preceding section indicated that the maximum lowering of  $\gamma$  was usually obtained at bulk concentrations below 1%, the following results were obtained with 1% solutions in the brombenzene for cholesterol and palmitic acid, and 10% with oleic acid

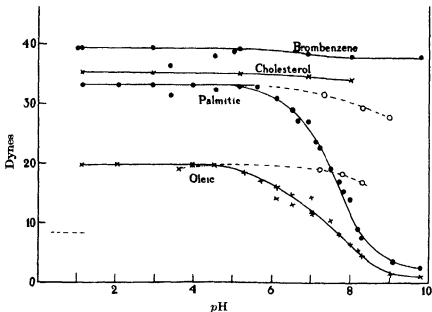


Fig. 2—Effect of pH on  $\gamma$  of solutions with nearly saturated surface layers, 0.4 M buffers

Fig. 2 shows the effect of pH, the buffers being, for the curves with the full lines, 0.4 M in cation. Over the range 1–4 of pH all full-line results refer to hydrochloric acid with sodium chloride added to bring the cation concentration up to 0.4 M. The two acid solutions gave the usual fall of  $\gamma$  between pH 5 and 9. For palmitic acid, the  $\gamma$ 's above pH 8.5 may be slightly too high, owing to the separation of solid soap at the interface

The neutral substances brombenzene alone and cholesterol gave a very slight fall with increasing alkalinity over about the same range. It is uncertain whether this is due to the substances themselves or to traces of acid impurities.

With the acetate buffers and still more in a few experiments not recorded on the curves with phthalate buffers there was a perceptible fall of  $\gamma$  increasing towards the acid side giving the dotted curves. This is probably due to the adsorption of undissociated acetic acid (cf. Adam 1936). Peters (1931) found a similar effect with phthalate buffers and Adam and Miller (1933) working with insoluble unimolecular films found evidence of an increase in surface pressure produced by adsorption of phthalic acid. These may be regarded as very simple cases of the film penetration recently studied by Schulman and Hughes (1935).

The broken curves are for glycine buffers made up without sodium chloride and therefore containing much less than 0.4 M cation. The fall in  $\gamma$  is much smaller at the same pH. At pH 8.5 the sodium concentration was about 0.01 M. It is evident that the concentration of cation is as important as the pH for producing this fall of  $\gamma$ .

With lecithin which is an amphoteric substance a few less accurate results were obtained the most striking characteristic of these being a maximum in  $\gamma$  between pH 6.5 and 7.5.

#### *c—Effect of Simple Dilution of Buffer Solutions*

Fig. 3 *a* shows the effect of dilution of the phosphate and borate buffers with distilled water on the  $\gamma$  against a 1% solution of palmitic acid. Occasionally the twentyfold dilution caused a change of pH of a few tenths of a unit and this has been taken into account in plotting the curves. The effect of dilution is to shift the curves parallel to themselves about 1.14 pH units for each twentyfold dilution. Fig. 3 *b* shows a shift for 10% oleic acid very similar in direction and magnitude. In the more alkaline range and the higher dilutions it was found that the amount of base removed by the oil phase or the interface made an appreciable difference to the pH and therefore the aqueous solution was usually renewed several times before measuring the  $\gamma$ . At more alkaline pH values than 8.5 the separation of solid sodium palmitate introduces a further inaccuracy into the measurements.

Figs. 3 *c* and *d* show that displacement of the curves was very small when the buffers were diluted with 0.4 M sodium chloride instead of water. The curves on the figures are taken from Figs. 3 *a* and *b*.



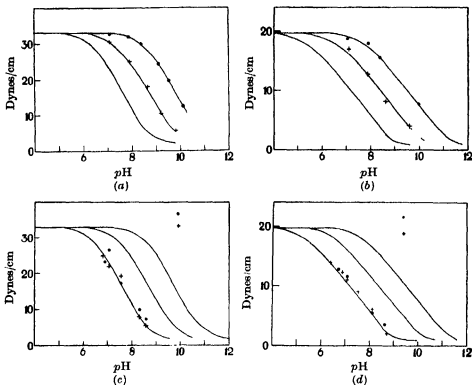


FIG. 3—*a* Effect of dilution of buffers with distilled water (1% palmitic acid) ● 0.001 M, × 0.02 M, — 0.4 M  
*b* Effect of dilution of buffers with distilled water (10% oleic acid) ● 0.001 M, × 0.02 M, 0.4 M  
*c* Effect of dilution of buffers with 0.4 M NaCl (1% palmitic acid) Curves from *a* ● diluted 900 ×, + diluted 20 ×.  
*d* Effect of dilution of buffers with 0.4 M NaCl (10% oleic acid) Curves from *b* ● diluted 400 ×, + diluted 20 ×.

*d—Effect of the Ratio Sodium Calcium ions*

Figs. 4 *a* and *b* show the effect of adding various concentrations of calcium chloride to glycine buffers, which were in every case 0.4 M in sodium. A few experiments were done with phosphate buffers at pH 6.9, with results rather similar to those with glycine at pH 7.2, but complicated and rendered inaccurate by the precipitation of calcium phosphate, work on more alkaline phosphate solutions was impossible for this reason. The logarithms of the calcium concentrations are plotted as abscissae, and the values for zero calcium have been inserted just to the right of the boundary of the figure.

The most noticeable fact is that, when the solutions are sufficiently alkaline, there is a large rise in  $\gamma$  with increasing calcium concentration, until the molarity of the calcium has reached roughly one-twenty-fifth that of the sodium. At pH 7.5 there is little change of  $\gamma$  on adding calcium; at 7.2 the 1% palmitic acid shows a moderate dip when the Na:Ca ratio

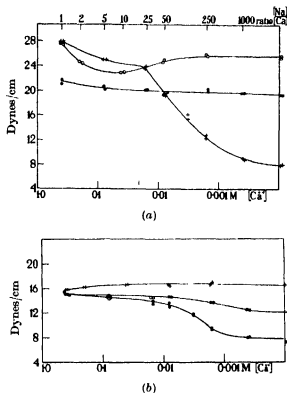


Fig 4—*a* Effect of addition of calcium to buffers initially 0.4 M in sodium (1% palmitic acid) ○ pH 7.2, ● pH 7.5, + pH 8.4  
*b* Effect of addition of calcium to buffers initially 0.4 M in sodium (10% oleic acid), + pH 7.2, ○ pH 7.5, ● pH 8.1

is about 5:1, but the 10% oleic does not. There was, with the phosphate buffers of pH 6.9, a similar dip both with the palmitic and oleic acid solutions.

With palmitic acid at pH 7.1 and at calcium concentrations greater than 0.05 M (Na:Ca ratio less than 8), a very curious periodic contraction and expansion of the surface was observed in one series of experiments, this observation could not, however, be repeated.

## DISCUSSION

The simple assumption that the amount of lowering of i.t. due to ionization is proportional to the extent of ionization, in the absence of complicating factors, accounts for the well-known similarity between the i.t.- $pH$  curves, and the curves of dissociation with  $pH$ . We have to find (a) the  $pH$  at the interface, and (b) an explanation of the shifts of the various S-shaped curves along the axis of  $pH$  when the cation concentration is varied.

The following treatment of these problems is not rigorous, and involves a number of assumptions which could not be checked quantitatively. For this reason the resulting calculations of surface  $pH$  are of qualitative importance only, and are of value mainly in that they indicate the order of

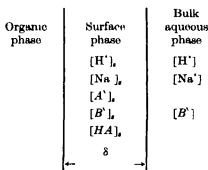


Fig. 5—Diagram of distribution of ions between surface and bulk phases

magnitude of the  $pH$  at a surface, and the manner in which this  $pH$  changes when certain types of change are made in the composition of the bulk phases. With more accurate data, more accurate calculations can be made by the same method. The assumptions are pointed out as they occur in the text, and are also listed at the end of this section (pp. 164-5).

A simple application of the Donnan equilibrium shows that the  $pH$  of a surface, where fatty acid molecules are held so that they cannot escape, will change as the concentration of cations in the aqueous phase changes. Fig. 5 represents an idealized view of the surface as a surface phase, of thickness  $\delta$ , with concentrations indicated by the suffix  $s$ . The concentrations in the bulk aqueous phase have no suffix.  $HA$  and  $A'$  refer to the undissociated acid and its anion, which are insoluble in the aqueous phase (the anion of oleic acid is slightly soluble at room temperature, but its solubility is here neglected as it is much smaller than the concentrations in

the surface phase).  $B'$  refers to the concentrations of the anion of the salt used for buffering the aqueous solutions

The Donnan equilibrium gives

$$\frac{[H^+]_s}{[Na^+]_s} = \frac{[H^+]}{[Na^+]}, \quad (1)$$

and the equilibrium between the acid  $HA$  and its ions in the interface is given by

$$[H^+]_s[A^-]_s = K_s[HA]_s, \quad (2)$$

where  $K_s$  is the dissociation constant in the surface. This assumes that a form of the mass-action law holds in the surface phase, similar to that in bulk phases, but does not assume that the dissociation constant ( $K_s$ ) in the surface is the same as that ( $K_a$ ) in the bulk phase

Combining (1) and (2),

$$[H^+]_s = \frac{[Na^+]_s[H^+]}{[Na^+]} = \frac{K_s[HA]_s}{[A^-]_s} \quad (3)$$

When the acid  $HA$  is 50% dissociated in the interface,  $[HA]_s = [A^-]_s$ , and

$$\frac{[Na^+]_s[H^+]}{[Na^+]} = K_s = [H^+]_s \quad (3.1)$$

Introducing the assumption that the additional lowering of  $\pi$  caused by electrolytic dissociation of the acid  $HA$ , below the  $\pi$  given by the undissociated acid, is proportional to the percentage of dissociation in the surface phase, then

$$\frac{F_r - F_0}{F_{100} - F_0} = \frac{r_s}{100} = \frac{[A^-]_s}{[HA]_s + [A^-]_s} \quad (4)$$

$F_0$  is the lowering of  $\pi$  produced by the undissociated acid,  $F_{100}$  by the completely dissociated acid, and  $F_r$  by the acid when  $r_s\%$  is dissociated

(4) gives the degree of dissociation of  $HA$  in the interface directly, from the interfacial tension, and thus the relation between  $r$  and the  $pH$  of the bulk phase, or  $[H^+]$ , can be found from the experimental data given in figs 2-3d

To calculate the hydrogen-ion concentration in the surface phase, probable values must be assumed for its thickness  $\delta$ , for the number of carboxyl groups per square centimetre, and the number of ions contributed to the surface layer by the buffer solution

The thickness of the surface phase is likely to be not less than 5 Å, nor more than 15 Å. The interfacial films are almost certainly "gaseous" films, under fairly high surface pressures and an area of 30 sq Å/mol will

probably be near the correct value \* The surface concentrations in moles per litre, corresponding to various thicknesses assumed for the surface phase, are shown in Table II In Table IIIa the necessary information is

TABLE II—VALUES OF THE SURFACE MOLARITY OF CARBOXYL FOR VARIOUS AREAS PER MOLECULE, AND VARIOUS ASSUMED THICKNESSES OF THE SURFACE PHASE

Area sq. Å.	Values of $\delta$		
	5	10	15
25	13.9	7.0	5.7
30	11.6	5.8	3.9
40	8.7	4.4	2.9

TABLE IIIa—CALCULATION OF THE DIFFERENCE IN pH BETWEEN THE SURFACE AND BULK PHASES FOR 1% PALMITIC ACID SOLUTION

$(pH)_b$ buffer	$r_s$	$[A']_s$	$[Na]_s$	$(pH)_s$ surface	$(pH)_b - (pH)_s$
Buffers 0.4 M in cation					
6	6	0.6	1.0	5.60	0.40
7	30	3.0	3.4	6.07	0.93
8	70	7.0	7.4	6.73	1.27
9	97	9.7	10.1	7.60	1.40
10	100	10.0	10.4	8.58	1.42
12	100	10.0	10.4	10.58	1.42
Buffers 0.02 M in cation					
7	4.5	0.45	0.47	5.62	1.38
8	24	2.4	2.4	5.92	2.08
9	65	6.5	6.5	6.49	2.51
10	95	9.5	9.5	7.32	2.68
11	100	10.0	10.0	8.30	2.70
12	100	10.0	10.0	9.30	2.70
Buffers 0.001 M in cation					
8	3.5	0.35	0.35	5.45	2.55
9	22	2.2	2.2	5.66	3.34
10	62	6.2	6.2	6.21	3.79
11	93	9.3	9.3	7.03	3.97
12	100	10.0	10.0	8.0	4.00

given for calculating the pH in the surface phase, and the difference between this pH and the pH of the bulk aqueous phase is also given.  $r_s$  is obtained from the experimental curves relating  $\Gamma$  to the pH of the aqueous

\* This value involves an extrapolation from the results on adsorbed films of slightly soluble substances, at air-water interfaces, to brombenzene-water interfaces. At air-water interfaces, the areas in such films are usually 30 sq Å or less, for a lowering of tension of 20 dynes. They may be greater at the brombenzene interfaces.

phase, using equation (4) From this, assuming the probable value of 10 M for the total concentration of HA and A' in the interface, the value of  $[A']_s$  is obtained. The amount of sodium ion in the interface is assumed to be equal to  $[A']_s$ , plus an extra amount due to the buffer. The extra amount due to the buffer is assumed to be the same as the amount present in an equal volume of the bulk aqueous phase From this data the pH in the surface phase can then be calculated, using equation (1)

TABLE IIIb—VALUES OF  $(pH)_s-(pH)_b$  FOR 10% OLEIC ACID SOLUTION.

pH buffer	Values of $(pH)_s-(pH)_b$ for cation molarity of		
	0.4	0.02	0.001
5	0.38	—	—
6	0.83	1.35	—
7	1.11	2.03	2.40
8	1.29	2.33	3.24
9	1.40	2.54	3.61
10	1.42	2.67	3.82
11	1.42	2.70	3.96
12	1.42	2.70	4.00

TABLE IIIc—VALUES FOR HEXADECYLAMINE SOLUTION OF  $(pH)_s-(pH)_b$  FOR BUFFERS 0.1 M IN ANION EXPERIMENTAL DATA FROM PETERS (1931)

$(pH)_b$	4	5	6	7	8
$(pH)_s-(pH)_b$	-2	-2	-1.96	-1.7	-1.0

As Table III shows, the difference between the pH values of the surface and bulk phases may amount to three or four units of pH, with the more dilute buffers, and in the physiological range of molarity the difference is of the order of two units. The nature of the molecules adsorbed at the interface is evidently an important factor for the determination of the surface pH. The values for the pH differences given in the tables are for the range covered by determinations of  $\gamma$ . Values outside this range can be calculated if assumptions are made as to the detailed form of the curve relating  $\gamma$  to pH, in the region where the degree of dissociation of the carboxyl groups in the surface phase is low, i.e. in the acid ranges.

#### DISPLACEMENT OF CURVES ON DILUTION

Equation (3) is 
$$[A']_s = \frac{[HA]_s K_a [Na^+]}{[H^+][Na^+]_s}$$

Substituting this value for  $[A']_s$  in (4),

$$F_s - F_0 = (F_{100} - F_0) \frac{[HA]_s [Na^+] K_a}{[H^+][Na^+]_s ([HA]_s + [A']_s)} \quad (5)$$

When the interface is saturated with  $HA$ ,  $([HA]_s + [A']_s)$  will not vary with degree of ionization, if it is assumed that the area per molecule does not change with ionization. This approximation is roughly correct at fairly high surface pressures.\*

Under these conditions (5) simplifies to

$$F_r - F_0 = BK_s \frac{[HA]_s [Na']}{[Na']_s [H']}, \quad (6)$$

where

$$B = \frac{F_{100} - F_0}{[HA]_s + [A']_s}$$

Consider equation (6). Decreasing  $[H']$  increases the value of  $(F_r - F_0)$ , i.e. decreases the  $\pi$ . Similarly increasing  $[Na']$  increases the value of  $(F_r - F_0)$ . But varying  $[Na']$  does not change the values of  $F_0$ , nor of  $F_{100}$ , nor does it affect the form of the equation relating  $(F_r - F_0)$  to  $[H']$ . Hence the effect of changing  $[Na']$  must be to displace the  $\pi$ -pH curve along the pH axis, but not along the  $\pi$  axis. This is just what was found experimentally (figs. 3a and b).

From equation (6) it can be seen that variation of  $[Na']$  has the inverse effect of varying  $[H']$ . Hence decreasing  $[Na']$  by, for example, twentyfold, should theoretically raise the  $\pi$  at a particular pH by the same amount as is produced by raising  $[H']$  twentyfold, i.e. the whole curve should be displaced by  $\log 20 = 1.3$  pH units. The value found experimentally was 1.14 pH units. The agreement between these two values is as good as can be expected, since many rather sweeping assumptions have been made. The main assumptions were (a) that a form of the mass action law holds in the surface phase, (b) that the amount of lowering of  $\pi$  due to dissociation is proportional to the amount of dissociation, (c) the use of concentrations instead of activities, (d) the thickness of the surface.

On figs. 3a and b are plotted curves of percentage change in  $\pi$  and dissociation in bulk solution against pH. The form of the  $\pi$  curves closely resembles that of the dissociation curve.

#### THE VALUE OF $K_s$

From equation (3) it can be seen that  $K_s$  is affected by the ratio of the cation concentrations in the surface and bulk phases. The simplest way to calculate  $K_s$  from the experimental data is to use (3.1), for which the data

\* Adam and Miller (1933) show that the area of palmitic acid in a unimolecular film lies between 20.5 and 25 sq. Å in the pH range 5-9. The areas at the oil-water interface are probably larger, but the percentage change is probably not larger.

needed are  $[\text{Na}^+]$ ,  $[\text{Na}^+]_s$ , and  $[\text{H}^+]$ , all at the point of 50% dissociation. These values are obtained by the same procedure as was used for the calculation of the pH at the surface. Thus for palmitic acid,  $[\text{Na}^+]$  is 0.4 M,  $[\text{Na}^+]_s$  is 5.0 M due to the palmitate ions plus 0.4 M due to the buffer, i.e. 5.4 M, and  $[\text{H}^+]$  from eq. 2 is  $3.2 \times 10^{-8}$ . Then from (3.1)

$$K_s = \frac{5.4 \times 3.2 \times 10^{-8}}{0.4} = 4.3 \times 10^{-7}$$

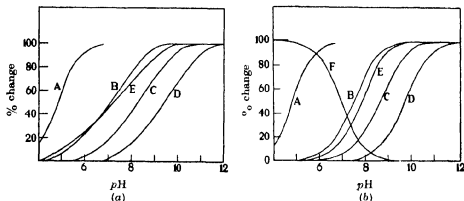


FIG. 6—*a* Curves of percentage change. A, approximate titration curve of oleic acid; B, C, D, 1% oleic acid, 0.4, 0.02, 0.001 M buffers; E, surface potential, myristic acid. *b* Curves of percentage change. A, approximate titration curve of palmitic acid; B, C, D, palmitic acid 1% 0.4, 0.02, 0.001 M buffers; E, palmitic acid half expansion temperature, 0.1 M buffers; F, hexadecylamine 1%.

Similarly for oleic acid,  $K_s = 10.6 \times 10^{-7}$ . A probable value for  $K_a$ , the dissociation constant of these acids in a bulk aqueous phase, is  $1.4 \times 10^{-5}$ . The value of the ratio  $K_s/K_a$  is, for palmitic and oleic acids respectively,  $3.1 \times 10^{-2}$  and  $9.3 \times 10^{-2}$ . These ratios show that the dissociation constants at the interface are less than that of the same substances in bulk aqueous solution. There are at least two reasons for this. Firstly, the dielectric constant of the interface is probably less than that of an aqueous phase, secondly, the carboxyl groups are close enough together for a repression of ionization to occur of the type suggested by Bjerrum (1923). The relationship of  $K_s$  to  $K_a$  will be of the form

$$K_s = K_a f\left(\frac{\epsilon^2}{dDKT}\right), \quad (7)$$

i.e. a generalized form of the equation for two carboxyl groups.  $\epsilon$  is the



electronic charge  $d$  the average distance between carboxyls,  $D$  the dielectric constant,  $k$  the gas constant, and  $T$  the absolute temperature

No absolute values for  $D$  have been obtained, but maximum values can be calculated from the surface potential of unimolecular films, these values suggest that  $D$  is of the order of 10 or less. A table of these maximum values taken from the work of Adam, Rideal, and their collaborators is given by Danielli (1935)

#### FURTHER POINTS

There are a number of other points in favour of the view that the  $\pi\tau$  changes considered here are due primarily to the ionization of the end groups of the surface active molecules

(a) A change in the molecules as drastic as ionization would be expected to cause a considerable change in  $\pi\tau$ . Hence if the changes considered above were not due to ionization then there should be a further change of  $\pi\tau$  over some other  $pH$  range which would be ascribed to ionization. Such a change is not known to occur

(b) With a fatty acid at the interface changing the nature of the cations in the buffer should influence the  $\pi\tau$  only over the  $pH$  range in which the acid is dissociated in the interface i.e. when there are anions in the surface to be affected specifically by cations. On the other hand, with an amine at the interface as in Peters's work, the effect of the anions of the buffers should predominate over the supposed range of ionization, i.e. the ion of opposite sign to that of the surface active material should exert a marked influence on the  $\pi\tau$  but only over the range in which the surface active substance is itself ionized. The experimental results are in agreement with this conclusion

(c) Hughes has determined the change of surface potential of unimolecular films of myristic acid with  $pH$ . This curve is plotted on fig 6a, the form of the curve and the range of the change of potential are similar to the changes of  $\pi\tau$  of the oleic acid solutions, plotted on the same figure

(d) Adam has investigated changes in the surface properties of amines in unimolecular films. The main changes in the packing of the molecules in the films occur over approximately the same range of  $pH$  as the  $\pi\tau$  changes with amine solutions investigated by Peters. Adam and Miller's work (1933) on unimolecular films of fatty acids similarly shows changes in packing over the same  $pH$  range as the change of  $\pi\tau$  with fatty acid solutions. The half expansion temperatures of the fatty acid films were also investigated. These are complicated by effects due to the anions of the

buffers on fig 6b data for the simplest case are given as a curve of the changes in half expansion temperature with  $pH$  for palmitic acid films on 0.1 M borate buffers the calculation has been made on the assumption that the number of  $pH$  units over which the change occurs is the same as that for  $\pi$  change with  $pH$  Adam and Miller also note that dilution of the borate buffers over this  $pH$  range has the effect of raising the half expansion temperature by about  $20^{\circ}C$  for a tenfold dilution According to equation (6) dilution of the buffers tenfold should shift the dissociation curve at the surface one  $pH$  unit to the alkaline side inspection of the half expansion temperature curve for the undiluted buffer shows that such a shift would raise the half expansion temperature by about  $20^{\circ}C$  in agreement with experiment

(e) The work of Deutsch (1927-1928) also suggests that  $pH$  differences exist between surfaces and surrounding bulk phases He found that when an indicator such as bromthymol blue in aqueous solution near its neutral point is shaken with a volume of an inert solvent such as benzene or toluene a colour change corresponding to a substantial change of  $pH$  occurs as the organic liquid is emulsified The solution reverts to its original colour as the emulsion breaks down With an acid dye the displacement is towards a more acid  $pH$  with a basic dye towards a more alkaline reaction Concerning the colour change with acid dyes Freundlich (1930) remarked This might be explained by assuming that the hydrogen ion concentration in the surface of the droplets is different from that in the bulk of the solution but this does not hold for if we take an indicator which is not an acid dye such as malachite green it also changes colour on being shaken with an inert fluid The change of  $pH$  which we would like to make responsible for the colour change has however a reversed sign in this case

However Freundlich and Deutsch evidently supposed that the difference between the interfacial  $pH$  and the bulk reaction was a pre-existing quantity existing prior to and independently of the adsorption of an indicator But as has been shown with the simple acids the adsorbed components of the interface are important variables in the determination of this  $pH$  difference The adsorption of an acid indicator at an interface will tend to produce a displacement of the  $pH$  at a surface to a more acid reaction just as a simple fatty acid does with a basic dye the  $pH$  change should be in the reverse direction Thus the results of Deutsch are consistent with the view that the  $pH$  of the surface phase is different from that of the bulk provided due account is taken of the fact that the indicator material is concentrated in the interface and that in consequence its

influence is not negligible as in the bulk phase, where the indicator will be in very dilute solution

(f) A somewhat parallel observation is that of Laing (1925), who found that the soap of films formed by adsorption from a sodium oleate solution was considerably hydrolysed much more so than the soap in bulk solution. This hydrolysis can be explained by the more acid reaction at the interface due to the high concentration of soap in the surface phase. Consistent with such a view it was found that the addition of a small amount of alkali would prevent this hydrolysis. A similar observation has been made by Dubrisay and Drouot (1933).

Adam (1936) has suggested that the acid soap formed by hydrolysis in bulk solution is less soluble and hence collects in the interface to a greater extent than the dissociated soap. This concept is not incompatible with the views suggested here which deal with the acid base equilibria with reference primarily to the surface phase. Adam's approach was primarily with reference to the acid base equilibria in the bulk phase.

#### INADEQUACIES OF THE PRESENT THEORY

The main defects of the present theory are (1) it does not consider the effect of divalent ions (e.g. calcium) (2) the theoretical relation of  $K_a$  to  $K_a'$  is not completely established (3) the effect of the anions of the buffer on the I.T. of fatty acid solutions is not taken into account. The half expansion temperature measurements of Adam and Miller show marked effects of buffer anions on films of fatty acids: this indicates that whilst the buffer ions of opposite sign to the surface active ions are of the greater importance the buffer ions of the same sign cannot be completely neglected.

#### SOME APPLICATIONS IN BIOLOGY

These surface pH phenomena may be of importance in biological systems. Professor J. C. Drummond has suggested that this may be particularly the case with intracellular enzyme reactions. The bulk of the fluid contents of the cell are presumably of fairly uniform reaction but the surfaces of granules, oil globules, mitochondria and gel particles may well differ from the bulk reaction by up to 2 pH units according to the constitution of the surfaces concerned. The cell may thus offer a much more diverse environment for enzyme reactions than has hitherto been supposed. The spatial range of these pH effects is small extending for a mere 5-10 Å from the physical

interface. A protein enzyme has a minimum diameter of probably 25 Å so if its active centres are to fall within the region of abnormal pH it must not only be adsorbed but also adsorbed with the correct orientation.

The results also suggest a reason for the changes which are observed in the behaviour of cells after washing with isotonic sugar solutions. Such a procedure reduces the cation concentration of the bulk phase to zero and thus according to equation (1) may produce very large pH changes at the cell surface changes which in turn may produce irreversible changes in the proteins of the cell surface. Evidence will be presented in a later paper that a surface pH change of one or two units is sufficient to change an adsorbed film of native protein into a denatured film with strikingly different properties.

Again this work shows that with for example an acid interface variation of the sodium concentration may produce an effect very similar to variation of the pH. It will thus be of interest to examine the effect of salts on the permeability of cells with the object of separating phenomena due to pH changes at the surface from phenomena which are essentially due to the specific action of cations as a case in point may be mentioned the effect of small amounts of electrolytes on the permeability of erythrocytes (Jacobs and Parpart 1932).

#### THE MECHANISM OF REDUCTION OF INTERFACIAL TENSION

There are a number of factors influencing the reduction of interfacial tension by an adsorbed substance so that it is difficult if not impossible to make a rule of thumb for the order of relative activity of substances in this respect. The main factors are

- (1) The number of molecules adsorbed per sq. cm
- (2) The lateral adhesion between the molecules
- (3) The area occupied by a molecule in the interface

The number of molecules in the surface will increase when one part of the molecule prefers to be in water and the other part in oil whereas when there is little differentiation between various parts of the molecules along these lines the tendency to accumulate at an interface will be minimized. Also there must be a balance between the dissolving powers of the two parts of the molecule since if the effect of one part predominates greatly, the molecule as a whole will have a high solubility in either the oil or the aqueous phase in which case the tendency to accumulate at an interface will again be minimized. It is partly for this reason that no one oil can be taken as standard in the sense that the order in which substances in a given

bulk concentration affect  $\gamma$  will remain the same with all other oils with other oils the partition coefficients are bound to be different and hence the number of molecules present in the interface for a given bulk concentration will be different. Thus while Traube's rule holds for an air-water interface within limited conditions the same rule cannot be applied to oil-water interfaces. As the interfaces of biological importance are usually oil-water ones and Traube's rule is used with more enthusiasm in biology than elsewhere it is of interest to quote an example from Peters (1931). He found that 1% palmitic acid reduced the benzene-water  $\gamma$  by 2.5 dynes whereas 1% lauric acid produced a fall of 3.7 dynes. If Traube's rule held the order would be reversed.

(2) The lateral adhesion between the molecules. If this is great the reduction in tension tends to be small and vice versa for a given number of molecules in the interface. This is the general rule found to hold at the air-water interface but again there is an additional factor present that is the hydrocarbon parts of the molecules are swimming in a sea of oil molecules (brombenzene in this work). With a gaseous film at the air-water interface there is only one position of minimum energy—that in which the molecules including hydrocarbon chains are lying flat on the surface. Probably that conclusion does not remain true when a sea of other hydrocarbon molecules is present and there may be many positions of minimum energy.

The importance of the lateral adhesion effects is shown particularly clearly by the changes in  $\gamma$  which occur on adding calcium to the phases surrounding an acidic interface. The large increase in  $\gamma$  found to the alkaline side of pH 8 is probably due to the powerful cross-binding forces introduced when one calcium ion is able to combine with more than one of the interfacial anions (fig. 7a). These cross-binding forces will be relatively small when univalent cations only are present and the large fall of  $\gamma$  found under these conditions is probably due to electrostatic repulsion and the heavy increase of hydration which accompanies ionization of the interfacial fatty acid. It would follow from this that when the surface is only slightly ionized the effect of calcium on the interface should be similar to that of a univalent ion since for spatial reasons a calcium ion will be able to combine with one interfacial anion only. The other anion neutralizing the calcium ion must be drawn from the bulk phase extra repulsive forces will be introduced by the third ionic layer which is formed by these anions (fig. 7b). These extra repulsive forces together with the fact that calcium ions are more heavily hydrated than the univalent ions may account for the fact that at pH values less than 7.5 calcium ions in

small amounts are sometimes even more efficient than univalent ions in reducing the  $\gamma$

(3) The area occupied by a molecule will depend on its size orientation the work required to change the initial orientation and amongst other things the amount of thermal motion of the molecules. The low surface pressure of cholesterol (i.e. small reduction of  $\gamma$ ) at saturation of the interface is probably to a large extent due to the fact that with only one hydroxyl group to each rather bulky molecule the interface must still rather largely consist of hydrocarbon since there is only one water attractive group to each 40 Å or more of the interface.

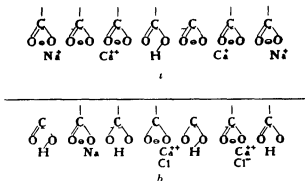


FIG. 7

It is a great pleasure to acknowledge my indebtedness to Professor E. N. Harvey in whose laboratory this work was carried out, also to Dr. N. K. Adam who has helped me considerably in preparing this paper and to Professor J. C. Drummond for a number of suggestions.

#### SUMMARY

The curves of variation of interfacial tension with  $pH$  for solutions of long chain acids in brombenzene are shifted parallel to themselves along the  $pH$  axis when the buffers are diluted with water. There is very little shift if sodium chloride solution is used for the dilution of such strength that the total cation concentration is not altered.

With alkaline solutions addition of calcium results in a very large increase in  $\gamma$  not found with neutral solutions. In certain circumstances with neutral solutions there is some degree of lowering of  $\gamma$  with increasing calcium concentration.

The effect of dilution is shown to be due to a Donnan equilibrium between

the interface and the buffers and a method is given for calculating the pH at the interface. The reaction at an interface in contact with physiological salt solution may be  $\pm 2$  pH units different from that of the solution. Some applications of this in biological fields are pointed out.

The mechanism of reduction of interfacial tension is discussed.

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# On the Electrical Responses of the Cochlea and the Auditory Tract of the Cat to a Phase Reversal produced in a Continuous Musical Tone

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[Plates 4-8]

## INTRODUCTION

The audible effects of changing by  $\pi$  ( $180^\circ$ ), the phase of a pure musical tone, have been described in a number of earlier publications (Hartridge 1921, 1936). Under certain conditions of frequency of the tone, and temporal spacing of the phase changes, the resulting sensation was found to be comparable to what Myers (1928) has described as the "rattling" stage of beats produced by two musical tones slightly out of unison. For this reason it has been considered justifiable to apply to the phenomenon the term "phase change beat". The possibility that this beat might be attributable to some transient disturbance in amplitude or frequency of the wave series other than the change of phase itself has been negated by a more recent investigation (Hallpike, Hartridge and Rawdon Smith 1936). In this the wave form of the phase change in the response from a loud speaker was assessed by means of a piezo crystal microphone, amplifier, and cathode ray oscillograph. By these means it was shown to be possible to elicit from the loud speaker a tone whose wave form was approximately sinusoidal, and which showed moreover at each change of phase, fair reproduction of the wave form of the impressed voltage. Even under such conditions the audibility of the "phase change beat" was found to be undiminished, thus justifying the view that the beat was attributable to the change of phase and to that alone.

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The work to be described in this paper concerns the investigation of the electrical events which take place in the mammalian (cat) internal ear and auditory tracts in response to such a phase change events which may be considered likely to correspond to the subjective sensation now described as the phase change beat. By such an investigation it has been hoped firstly to throw light upon the physical nature of the vibrating elements of the internal ear responsible for the initiation of the nervous impulses the action potentials of which can be recorded in the manner first described by Davies and Saul (1932) in the auditory tracts of the mid brain and further by a comparison of the response to a phase change of these tract potentials with the corresponding response of the cochlear potentials described by Wever and Bray (1930) to elucidate the still obscure problem of the latter's origin.

#### APPARATUS

A schematic representation of the experimental arrangements is shown in fig. 1. These may be described under the following headings: 1—Sound generation, 2—Amplification and recording, 3—Monitoring.

##### 1—SOUND GENERATION

The device employed has been derived from one already described (Hartridge 1936). In its original form the apparatus consisted of a brass disk of some 15 in. in diameter in the periphery of which 100 radial slots were cut, each 1 mm. in width by 10 mm. in length. A jet of compressed air was arranged to play upon the slots of the disk whilst in rotation, thus constituting a tone generator of conventional siren type. Intensity and frequency were controlled respectively by variation of air pressure and of the speed of rotation of the disk. Further, by adjustment of the form of the air tube nozzle, some approximation to sinusoidal wave form was found possible. The abandonment of this method was, however, necessitated by the marked high frequency components engendered in part by the impaction of the air blast upon the edges of the slots, and in part by random eddy currents about the orifice of the jet. These high frequency components were eliminated by replacing the air jet by a light beam by which a copper oxide photocell was illuminated via the slots in the rotating disk (Hallpike and others 1936). Later, a cardboard disk was employed with a series of spokes cut upon its periphery to interrupt the light beam as shown in fig. 2. As indicated here, the light was disposed to give an approximately parallel beam, and by means of a suitable stop, the exposed portion of the



photocell was arranged to consist of a circle of diameter corresponding roughly to the cross-section of the light beam and more precisely to the transverse dimension of the spokes and interspoke gaps. The theoretical wave form of the photocell output under these conditions was readily calculable by plotting upon a time base the areas of the photocell face successively exposed to the light beam, and, further, this calculated wave form was found to exhibit fair correspondence to the form actually obtainable as shown in fig 3, A and B, Plate 4. By suitable variation in the size of the spokes, a close approximation to sinusoidal wave form was found to be possible, the optimum arrangement being depicted in fig 4, Plate 4. The phase change was effected in a simple manner by breaking the alternation of spoke and gap at any point upon the circumference of the disk by two successive spokes. To change the phase in the opposite half of the cycle it was similarly required to break the spoke/gap alternation by two successive gaps.

The output from the photocell was fed directly into the grid-cathode circuit of a triode amplifying stage. It was not found desirable to attempt to match the low impedance of the cell to the high-grid input impedance of the valve for two reasons. First, because certain minor distortions of wave form were introduced by the iron-cored transformer tried in this position, particularly at the change of phase, and secondly, because the presence of the latter rendered the input circuit of the amplifier somewhat too sensitive to electromagnetic disturbances from the motor employed to drive the rotating disk.

The input stage was succeeded by two stages of conventional resistance-capacity coupled triode amplification, feeding a third stage, choke-coupled to a General Radio 60 decibel L-section attenuating network. By means of this instrument, the amplitude of the input to the succeeding power stage could be conveniently controlled. The power stage itself consisted of a pair of 25-W triodes, operating in push-pull. The output from these was fed through a specially designed transformer to the L S 4 moving-coil loud-speaker unit employed for acoustic stimulation.

Final adjustment of the precise wave form of the phase change was made to suit the response of the loud-speaker at the frequencies employed. Thus, fig 5, Plate 5 shows a microphone record of the response of the loud-speaker to an unsuitable electrical input. The response to a more suitable input is shown in fig 6, Plate 5, together with the disk contour employed in its production. At lower frequencies, the difficulties of eliciting from the loud-speaker a reproduction of the phase change free from marked transient disturbances of amplitude became considerably greater. Fig 7, Plate 5

shows the irregularities which occur at 256 ~ in response to an electrical input of wave form similar to that which gave a satisfactory response at 1024 ~

It was found possible to counteract this irregularity in amplitude of the loud speaker response by suitable modification of the corresponding waves of the electrical input. This was effected by constructing the contour of the disk of coloured gelatine at and about the position of the phase change the colour and thickness of the gelatine being varied as required.

The wave form of the electrical input to the loud speaker required to produce a relatively smooth phase change at 256 ~ is shown in fig. 8 Plate 5 with the corresponding loud speaker response.

## 2—AMPLIFICATION AND RECORDING

In order to amplify the potentials derived from the cochlea or mid brain of the experimental animal we have employed the conventional multi stage resistance capacity coupled amplifier described earlier (Hallpike and Rawdon Smith 1934 *a*). A similar instrument is employed following the piezo electric microphone with which wave form monitoring is carried out. The outputs from these two amplifiers are fed to two cathode ray oscillograph tubes of identical type (Cossor 3232 Screen Type C) so arranged as to permit their simultaneous recording on 35 mm. oscillograph bromide paper. For full particulars of the mechanical and optical arrangements comprising the camera mechanism reference may be made to a separate publication (Hallpike 1936).

## 3—MONITORING

Frequency and wave form monitoring devices have been employed in the following way. A cathode ray tube additional to the two already described and operating in conjunction with a synchronizable linear sweep (Hallpike and Rawdon Smith 1934 *a*) may be connected at will into (*a*) the input circuit of the stimulating loud speaker (*b*) the output of the piezo microphone amplifier or (*c*) the output of the amplifier connected to the cat.

In this manner the wave form of the stimulus or of the response may be readily observed simultaneously if necessary with the taking of photographic records. For frequency monitoring the linear sweep is replaced by a sinusoidal voltage derived from the time controlled 50 ~ A.C. mains. Any frequency which is in simple ratio to 50 ~ /sec. may thus be secured with amply sufficient accuracy for our purposes by inspection of the Lissajous figure appearing on the oscillograph screen.

In addition to this system two separate loud speakers are provided for monitoring the sound input to and electrical response from the experimental animal. By means of these frequency monitoring against standard tuned tuning forks may also be employed where rapidity of operation is important.

#### EXPERIMENTAL METHODS

Following decerebration under ether anaesthesia the animal's head was immobilized in the lateral position by means of a head holder working upon a universal joint and providing the necessary grip by means of a pair of screw clamps operating upon the zygomatic arches which were exposed by preliminary dissection. The tympanic bulla was opened by the usual neck dissection and the region of the lateral fillet exposed to view by partial resection of the bony tentorium and reflexion of a flap of dura mater. A small brass tube was tied into the stump of the external auditory meatus for the attachment of the sound tube. In the electrode system used for recording from the tract the active electrode consisted of a fine stainless steel needle insulated excepting at the tip with a layer of bakelite varnish. The indifferent electrode consisted usually of a large platinum ball applied to an adjacent portion of the brain stem. The electrodes were carried upon modified micro manipulators the active electrode being applied under direct vision to the superficial portion of the brain stem lying between the inferior *corpus quadrigeminum* and the trochlear nerve. For recording from the round window an active electrode of platinum applied to the round window margin was employed in conjunction with an indifferent electrode in the neck muscles. For simultaneous recording from the round window and tract two separate input systems were used in accordance with the circuit suggested by Ross (1935). Otherwise a common earth system was employed for the indifferent electrodes.

#### RESULTS

Figs 9 10 11 and 12 Plate 6 show some of the records obtained in a series of eight cats of the electrical responses from the auditory tract and round window to 180° phase changes. The frequencies employed were 256 ~ and 1024 ~ at sound intensities varying between 40 and 80 phons. It will be seen that the tract responses to the phase change are characterized in all cases by a marked silent period of two or three cycles following which the response rapidly reassumes its original amplitude.

In fig 13 Plate 6 is shown a similar record obtained in one other animal of the series in which the response differs materially from those

depicted in figs 9, 10, 11 and 12, Plate 6, in that the silent period is absent, though abnormalities in respect of latent period or 'on' effect were not noted (Davis, Derbyshire Lurie and Saul 1934)

In the case of the cochlear phenomenon which has been recorded from the round window, the response is seen to be characterized in all cases by the reproduction of the impressed phase change with varying, but generally considerable, accuracy

#### DISCUSSION

The nature of the responses thus recorded in the case of the auditory action potentials and of the Wever and Bray phenomenon justify a number of observations bearing upon the physical characteristics of the vibrating elements wherein arise the electrical phenomena concerned. The silent period exhibited by the tract response strongly accords with the view that the vibrating elements which are responsible for the initiation of these action potentials are resonant structures, the silent period being brought about by the opposition of the forces operating after the phase change to the after swings caused by resonance. There seems little reason to reject the widely held view that the resonant structures in question are the fibres of the basilar membrane. Further, these findings are in entire agreement with the view previously advanced by Hartridge (1921) that the phase change beat engendered in the auditory experience of the human observer has as its basis the silent period now shown to occur in the auditory tract response of the cat.

In the case of the anomalous type of response depicted in fig 13, Plate 6, the possibility arises that such a result may be attributable to factors concerned with electrode position. Thus, assuming that frequency localization is maintained at the level of the lateral fillet, and that further, following the phase change, there occurs at the same time as the silent period of the in tune resonators a transient response from various out of tune elements, then an electrode in a position corresponding to the latter might be expected to give some such response as is shown in the figure. Under these circumstances, the smaller amplitude of the out of tune elements would be compensated by the electrically advantageous position of the electrode relative to these elements and the amplitude of the wave series following the phase change would thus tend to be maintained. This possibility seems to be excluded by the experimental finding that at this level of the brain stem, frequency localization does not appear to be maintained. Thus in any one of the cases shown in figs 9-12 Plate 6, the records for both low and high frequencies (256 ~ and 1024 ~) were taken from the same

electrode position. Reference may here be made to the procedure adopted for determining the optimum electrode position for recording from the tracts. a number of electrodes are set up in a variety of positions covering the region of the lateral fillet. By means of a multi-change switch these are led in turn to the grid lead of the amplifier and the electrode position yielding the optimum response is in this way rapidly determined. The variation in amplitude of the responses obtained from such a selection of electrodes is considerable and must depend upon factors other than frequency localization. As stated, the optimum electrode position, once determined, exhibits no frequency differentiation.

By way of comment upon the responses shown in fig. 13, Plate 6, it is only possible to say that the potential changes appear in this case to arise in highly damped non-resonant structures. Reverting to the relationship of the phase change beat to the silent period otherwise shown to be typical of the tract response, it appears by no means certain that the gap in the response described as the "silent period" need of necessity be regarded as an indication of complete physiological inactivity. It may well correspond to a burst of unsynchronized impulses initiated by out-of-tune elements which, though beyond the resolving power of the recording apparatus, may yet be of significance sufficient to constitute the physiological counterpart of the phase change beat.

It may be noted that such a transient disturbance of out-of-tune elements evoked by a phase reversal would be in theoretical accord with the physical properties of a resonator system such as is postulated to exist in the cochlea. (In such a system, transient oscillations would in accordance with theory be set up in out-of-tune elements at the onset of a single musical tone. phase reversal is mathematically equivalent to a sudden superimposition upon a musical tone, of a second tone of identical frequency, of double its amplitude and differing in phase by  $180^\circ$ . A disturbance of out-of-tune elements similar to that occurring at the onset of a single tone would therefore be expected to occur at a phase reversal.) It must be added that the records of the tract responses (figs. 9-12, Plate 6) indicate clearly that the amplitude attained by such out-of-tune elements in forced response to the phase change falls greatly below that of the free response of the in-tune elements.

It may be taken that in a vibrating system of this type the extent to which the free response of the in-tune resonators is approached by the transient activity of out-of-tune elements occurring in response to the phase change must depend upon the sharpness of tuning of the elements concerned. Thus the record depicted in fig. 13, Plate 6 can be regarded as

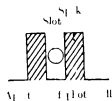
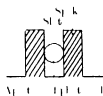


Fig. 3

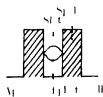
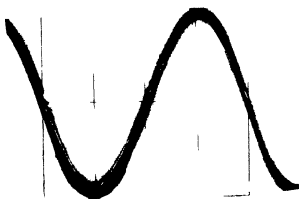


Fig. 4



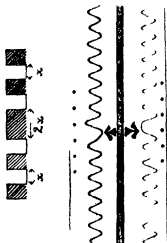


FIG. 7

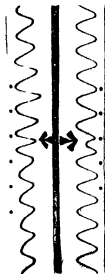


FIG. 8

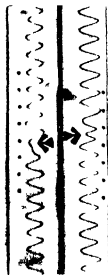
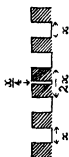


FIG. 6



FIG 9a



FIG 9b

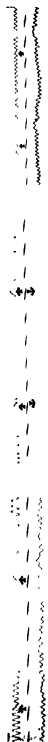


FIG 9d

FIG 10a

FIG 9e



FIG 11a

FIG 10b

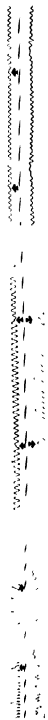


FIG 11c

FIG 11b

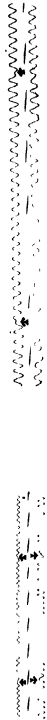


FIG 12a

FIG 13

FIG 12b



being derived from a system in which any tuning might be considered negligible or absent. A comparison of this record with the tract responses shown in figs 9 10 11 and 12 Plate 6 may be taken as marking the extent to which sharpness of tuning has been attained by the physical elements in which the latter responses originate.

As already described the responses from the round window are typified in all cases by a more or less faithful reproduction of the phase change. The cochlear effect must upon this evidence be regarded as originating in some heavily damped non resonant structure. This would appear to enforce considerable modification of prevailing opinions upon the subject. According to the view at present most in favour the cochlear component of the Wever and Bray phenomenon originates somewhat in the manner of a piezo electric effect in the hair cells of Corti's organ. This view has been put forward by Davis and his co workers in a number of publications (Davis and others 1934 Davis 1935). The following considerations prompt the belief that this possibility must now be regarded as definitely excluded. All available evidence supports the view that the basilar membrane is composed of resonant elements; further the hair cells are directly attached to these elements. Thus a resonant type of response to a phase change is clearly necessitated on the part of the cochlear response if the hair cell theory of its origin is to remain tenable. The evidence now adduced provides clear proof that the cochlear response does in fact arise in structures in which resonance is absent. It cannot therefore arise in any structure (including the hair cells) which is connected with the basilar membrane. The possibility that the cochlear response originates in action potentials in the terminal fibres of the cochlear nerve has been largely negated by the findings of Davis and his collaborators (Davis and others 1934). Though as indicated in a previous publication (Hallpike and Rawdon Smith 1934*b*) some of Davis's arguments are inconclusive his experimental finding that the polarity of the response is dependent upon the phase of the stapedial movement remains as a very cogent contra indication and one moreover which we have found to be readily confirmed. The present experimental evidence strongly favours the view that the terminal cochlear fibres as being structures connected with the resonant basilar fibres cannot be concerned in the origin of the cochlear response in view of the latter's conspicuously non resonant type of response to a phase change. Apparent support for the action potential view of origin of the cochlear response has been provided (Hallpike and Rawdon Smith 1934*b*) by the demonstration of an absent response in a denervated but otherwise morphologically normal cochlea. Further consideration of the interpretation of these

findings, together with additional morphological data, are to be found in the succeeding paper. It remains to consider the bearing of the present experimental evidence upon the membrane potential theory of origin of the response. As originally put forward (Hallpike and Rawdon-Smith 1934a), this theory attributed the response to movements occurring in a polarized membrane. In particular, the membrane of Reissner was cited, with data suggesting the probable mechanism of polarization. In so far as the markedly non-resonant type of response to a phase change shown to be typical of the response is in clear accordance with the physical characteristics of Reissner's membrane (*viz.*, small mass and heavy damping), the present experimental data must be considered as strongly favouring this or some similar structure as being the probable source of origin of the phenomenon. For further morphological evidence in support of this view, reference may be made to the succeeding paper.

#### SUMMARY

The constructional details and the mode of operation are described of a photoelectric siren which has been employed for the production of phase changes of  $180^\circ$  in continuous tones of good wave form.

The electrical responses elicited by this form of stimulation from the auditory tract and round window of the decerebrate cat have been recorded and are described, together with the technical methods employed.

In the case of the auditory tract, the response is described as being of a resonant type in that it displays a well-marked "silent period" following the phase change. This is thought to confirm the view that the moving parts concerned in the initiation of the cochlear nerve potentials are resonant structures. The probability that these structures are the fibres of the basilar membrane is accepted. The findings are further thought to confirm fully the suggestion previously put forward (Hartridge 1921) that the sensation engendered by the phase change and now described as "the phase change beat" has as its physiological counterpart a silent period as now shown to occur in the tract response of the cat.

The response of the cochlear component of the Wever and Bray phenomenon, as recorded from the round window, is found to be of a non-resonant type, in that no silent period occurs and the phase change is reproduced with marked accuracy. This is considered to provide strong evidence in favour of the membrane potential theory of origin of the cochlear response. The action-potential theory, as also the hair-cell theory of Davis and his collaborators, are further considered to be definitely excluded by the data provided.

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## DESCRIPTION OF PLATES

## Plate 4 \*

FIGS 3 and 4—Photographic enlargements of oscillograph records showing wave forms obtained with the conditions of illumination as depicted in the appended diagrams. The degree of correspondence is shown with the form theoretically obtainable (continuous fine line) Sine wave interrupted line. In fig 4 the theoretical and sinusoidal forms correspond very closely. The latter form is not represented separately.

## Plate 5

FIGS 5, 6, 7 and 8—The lower tracing in each figure represents the amplified electrical output of the photo cell. The upper tracing is in each case a microphone record of the corresponding loud speaker response.

FIGS 5 and 6—Frequency 1024~. Diagrams are superimposed of the disk contours employed.

FIGS. 7 and 8—Frequency 256~.

## Plate 6

FIGS 9, 10, 11 and 12—Oscillograph records of auditory tract and cochlear responses to 180° changes of phase. In all the records the upper tracing is of the microphone response. Arrows indicate the position of the phase changes. Time marker 1/200th sec.

FIG 9—Cat No 1 a 256~ Microphone and tract b 256~ Microphone and cochlea c 1024~ Microphone and tract d 1024~ Microphone and cochlea.

FIG 10—Cat No 2 a 1024~ Microphone and tract b 1024~ Microphone and cochlea.

FIG 11—Cat No 3. a 256~. Microphone and tract b 1024~ Microphone and tract c 1024~ Microphone and cochlea.

FIG 12—Cat No 4 a 1024~ Microphone and tract b 1024~ Microphone and cochlea.

FIG. 13—Oscillograph records of auditory tract response to 180° phase changes. Cat No 5 Frequency 1024~. The upper tracing is of the microphone response. Arrows indicate the position of the phase changes.

# On the Changes in Histological Structure and Electrical Response of the Cochlea of the Cat following Section of the VIIIth Nerve

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[Plates 7–12]

## INTRODUCTION

According to Witmaack (1911), division of the VIIIth nerve in the cat results in widespread degeneration of the cochlear neurones peripheral to the point of section, the morphological integrity of all other cochlear elements being maintained. These findings have been confirmed by later observers (Kaida 1931, Hallpike and Rawdon-Smith 1934 *b*), who have in addition shown that these results require for their production the avoidance of damage to the cochlear vessels, which is found to bring about a generalized necrosis of the contents of the internal ear. A study of the electrical response of the internal ear (cochlear component of the Wever and Bray phenomenon) found to occur in the cochlea of a cat denervated in the manner described, has been the subject of a previous publication (Hallpike and Rawdon-Smith 1934 *b*). In this, absence of electrical response was found to occur in a cochlea morphologically normal apart from the elimination of the neural elements. The results of a further series of observations upon these lines are now available and form the subject of the present paper.

## METHODS

Under nembutal anaesthesia the VIIIth nerve was divided in a series of fifteen cats. A ball-pointed dental probe was passed blindly into the

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‡ Working with a grant from the Medical Research Council, and later, Senior Student of the Royal Commissioners of the Exhibition of 1851.

internal auditory meatus and the nerve divided by gentle pressure. The operative mortality was nil, and no septic or other complications were encountered.

Acute vestibular disturbance was manifested in all cases during the week following the operation. Head rotation and facial palsy were more persistent and varied in degree.

The electrical response of the cochlea was assessed in each case at periods varying from 2 days to 23 weeks after the operation. This was carried out in the following manner: after decerebration under ether anaesthesia, both tympanic bullae were opened by the usual neck dissection, and recording electrodes of platinum placed in position upon the round window margins together with an earthed electrode in the neck muscles. The recording electrodes were connected via a change-over switch to the grid lead of an amplifier (resistance-capacity coupled of conventional type) feeding a cathode-ray oscillograph. The maximum amplitude of the responses obtainable from the round window at frequencies of 256, 512, 1024, and 2048 cycles was measured directly upon the tube face and matched in each case by injecting sinusoidal potentials of corresponding frequencies generated by a heterodyne oscillator and introduced into the input circuit via a calibrated potentiometer. The measurement of the voltages injected in this way, and matching the peak voltage values of the corresponding cochlear response was made with an A C (copper-oxide) voltmeter, connected across the outer terminals of the potentiometer. In all cases the reflex response of the tympanic muscles to auditory stimulation was observed to be absent upon the side of operation, stimulation of the opposite ear resulted in all cases in a brisk response. *Intra-vitam* fixation was immediately carried out, Ringer's fluid followed by Witmaack's solution being injected into the aorta, with a counter opening in the right ventricle. The solutions were injected at a temperature of 37° C and pressure of 80 mm Hg. The temporal bones and brain stem were removed, and, after further hardening and decalcification, embedded in celloidin. Serial sections were made in the horizontal plane at 20  $\mu$ .

## RESULTS

In four members of the series, gross necrosis of the cochlear contents was found to have occurred as depicted in fig. 1, Plate 7. They have accordingly received no further consideration in the present paper. The remaining members have been classified in accordance with histological findings into three groups.



*Group I*

The histological changes found in the members of this group are given in detail in Table I, together with the electrical responses obtained. The findings characterizing this group may be described as follows

i—The neural elements exhibit varying but, in general, considerable degrees of disintegration

ii—Corti's organ, and in particular the hair cells, shows well-marked changes, in two cases, hair cells approximating to normal are present only in the basal whorl

iii—The electrical responses may be described as being either normal (considered in respect to that obtained from the opposite intact ear), or closely approaching thereto

The changes found in Case No 147, which may be considered typical of this group are depicted photomicrographically in figs 2-9, Plates 7, 8, 9

*Group II*

The histological changes found in the members of this group are given in detail in Table II, together with the electrical responses obtained

The findings characterizing this group may be described as follows

i—The neural elements exhibit an advanced degree of disintegration.

ii—Corti's organ, and in particular the hair cells, is relatively well preserved In cases No 149 and No 150 no significant departure from normality can be observed

iii—Organized exudate occurs in small amounts in the scala tympani adjoining the opening of the cochlear aqueduct

iv—The electrical responses are absent or greatly reduced

The changes found in case No 150, which may be considered typical of this group, are depicted photomicrographically in figs 10-17, Plates 9, 10, 11

*Group III*

The histological changes found in the members of this group are given in detail in Table III, together with the electrical responses obtained The findings characterizing this group may be described as follows

i—The neural elements exhibit early degenerative change Cloudy swelling of the cells of the spiral ganglion with nuclear disintegration is generally to be noted

ii—Corti's organ, and in particular the hair cells, is well preserved.

iii—There is a well-marked serous labyrinthitis extending throughout

the cochlea. Coagulum formation is to be seen in all the perilymph spaces, and in one case a considerable haemorrhage has occurred.

iv—The electrical responses are found to be either normal or enhanced with respect to that obtained from the opposite intact ear.

The changes in case No. 130 which may be considered typical of this group, are depicted photomicrographically in figs 18–22. Plates 11, 12, 13

#### DISCUSSION

The results given in Tables I and II may conveniently be considered together. They are of interest chiefly for the evidence they provide bearing upon the hair cell theory of origin of the Wever and Bray phenomenon propounded by Davis and his collaborators (1934). According to this theory the phenomenon is regarded as arising somewhat in the manner of a piezo electric effect from the hair cells of Corti's organ. The evidence now provided shows firstly (Table I) the possibility of a normal electric response occurring in association with marked degenerative changes in the hair cells. In Table II the converse possibility is established, namely, of a much reduced electric response occurring in association with morphologically normal hair cells. It is considered in the light of this evidence that the hair cell theory of origin of the Wever and Bray phenomenon must be regarded as untenable.

The results described in Table III may conveniently be considered in association with the findings in certain other animals of the series in which labyrinthitis is a marked feature.

The cases are divided into two groups depending upon the length of the survival period.

In the cases with survival times of 5 weeks and under the labyrinthitis is of severe degree and is more pronounced upon the side of nerve section. In this group may be placed the following:

Table I, No. 130, No. 124.

Table II, No. 123. In this case a gross haemorrhage has occurred in addition to the labyrinthitis.

Table III, No. 131, No. 129, No. 125.

In cases with longer survival times (12 weeks and over) the labyrinthitis has to a large extent undergone resolution, with the exception of organized exudate occurring in the scala tympani around the opening of the cochlear aqueduct. In this group may be placed the following:

Table I, No. 161.

Table II, No. 149, No. 150.

TABLE I

No of animal	Survival time		Wever and Bray response microvoluta (Peak)		Histological findings	
	Weeks	Days	Left ear	Right ear	Left ear	Right ear
130	1	3	510	256 ~	640	900
			790	512 ~	1010	1024 ~
			1010	1024 ~	1120	2048 ~
						560
124	4	1	660	256 ~	940	770
			1200	512 ~	1100	880
			780	1024 ~		
			1200	2048 ~		
122	6	3	320	256 ~	700	700
			200	512 ~	1000	700
			200	1024 ~		
			275	2048 ~		

Marked retraction of the tectorial membrane has occurred at the apex. Thus Reuser's membrane are otherwise normal. Considerable disintegration of Corti's organ is found. Thus, in the lateral middle whorl all cellular elements other than Corti's rods have disappeared. Earlier stages of the disintegration are visible in the internal middle and apical whorls. Preservation of the nuclei of the hair cells and Deiter's cells is found only in the basal whorl, though here also changes in the cell bodies leading to obscuration of the cell outlines are marked. Early degeneration has taken place in the neural elements. This is shown chiefly in the cloudy swelling and nuclear fragmentation which has occurred in the cells of the spiral ganglion. Some reduction in the number of these is to be noted. There is a marked serous labyrinthitis, indicated by fine exudate in all the perilymph spaces.

Reuser's and the tectorial membrane are normal. Gross changes have occurred in the cells of Corti's organ. In the apical whorls these have undergone detachment from the basilar membrane. In other whorls the hair cells and Deiter's cells have lost their nuclei. Cloudy swelling of the cell substances has caused conspicuous loss of the cell outlines. In the internal basal coil, and here alone, the structure of the organ of Corti is found to be normal, apart from the absence of nerve fibrils crossing the tunnel of Corti. There is a slight but definite labyrinthitis as shown by exudate scattered throughout all the perilymph spaces.

The tectorial membrane has become detached in places. Reuser's membrane is normal. Gross changes have occurred in Corti's organ. Thus in the internal apical whorl, total disintegration and disappearance of the cellular elements have occurred. An earlier stage of this process is exhibited in the lateral apical whorl. Elsewhere, loss of the nuclei has occurred in the hair cells and Deiter's cells. In the internal basal whorl these nuclei are retained, though here as elsewhere changes in the cell protoplasm have led to marked loss of cell outline. The neural elements show conspicuous alteration. A considerable decrease has occurred in the number of medullated fibres in the nodulus and osseous spiral lamina as also in the number of the cells of the spiral ganglion. A slight serous labyrinthitis is present as indicated by the presence of exudate in some of the perilymph channels.

Scattered traces of exudate occur chiefly in the perilymph spaces, indicating a very slight degree of serous labyrinthitis. The cochlea is in all other respects normal.

There is a very slight degree of serous labyrinthitis as indicated by traces of exudate occurring in the perilymph spaces. The cochlea is in all other respects normal.

Normal.

147	14	3	210	256 ~	180	Slight bulging of Resener's membrane is seen in all whorls. The tectorial membrane is normal. Well-marked changes have occurred in Corti's organ. The framework of Corti's organ is everywhere preserved. The cells of Deiter and the hair cells have in many places lost their nuclei. Thus together with a marked loss of cell outline has converted the cells in places into masses of comparatively undifferentiated protoplasm. In the internal basal coil, and here only, normal structure of Corti's organ is preserved. Considerable changes have occurred in neural elements. The number of medullated fibres in the modiolus and osseous spiral lamina is much reduced. A few degenerate cells of the spiral ganglion are found towards the apex with a small better preserved clump towards the base. A well marked serous labyrinthitis has occurred with exudate in the perilymph channels.
			220	512 ~	230	
			260	1024 ~	300	
			220	2048 ~	200	
161	22	6	50	256 ~	440	Resener's and the tectorial membrane are normal. The framework of Corti's organ including the rods of Corti is preserved. The hair cells and Deiter's cells have lost their nuclei. Loss of cell outline is marked and the cell bodies appear in large part as an undifferentiated mass of protoplasm. In the internal basal coil, and here only, the organ of Corti is of normal appearance apart from the absence of nerve fibrils crossing the tunnel of Corti. Changes in the neural elements are widespread and of extreme degree. Apart from a few scattered and degenerate cells occurring towards the apex, the spiral ganglion has undergone complete degeneration. Serous exudate occurs in small amounts scattered about the perilymph spaces indicating a mild degree of serous labyrinthitis.
			70	512 ~	440	
			90	1024 ~	580	
			100	2048 ~	320	

There is a marked serous labyrinthitis with exudate in the perilymph channels. The cochlea is in all other respects normal.

There is a very small amount of serous exudate in the scala tympani of the basal whorl. The cochlea is in all other respects normal.

TABLE II

No of animal	Survival time		Wever and Bray response microvolts (Peak)		Histological findings	
	Weeks	Days	Left ear	Right ear	Left ear	Right ear
123	4	5	40	256 ~	Reissner's and the tectorial membrane are normal. Apart from the absence of nerve fibrils crossing the tunnel of Corti the cells and framework present an entirely normal appearance throughout the cochlea. Advanced changes have occurred in the neural elements. The nerve fibres in the modiolus and the osseous spiral lamina are much reduced in number. There are a few degenerate cells representing the remains of the spiral ganglion. The spaces of Rosenthal's canal being otherwise occupied by neurolemmal remnants. There is a slight degree of serous labyrinthitis with scattered exudate in the perilymph channels. In addition a considerable haemorrhage has occurred into the apical portion of the scala vestibuli.	Exudate and pigment granules occur in small quantities scattered about the perilymph spaces indicating a recent mild serous labyrinthitis. The cochlea is in all other respects normal.
149	13	1	—	256 ~	Reissner's and the tectorial membrane are normal. No abnormality can be observed in the arrangement or appearance of the cells of Corti's organ in any part of the cochlea, apart from the absence of nerve fibrils crossing Corti's tunnel. Gross and widespread degeneration of the neural elements has occurred. Very few of the medullated fibres occupying the modiolus and osseous spiral lamina remain. The spiral ganglion is represented by a few degenerate cells towards the apex. Evidence of a slight degree of serous labyrinthitis is provided by small coagula appearing in the scala tympanum of the lateral basal whorl also upon the lower surface of the basilar membrane in the internal middle whorl.	A small haemorrhage and some exudate is found in the scala tympanum of the basal whorl. The cochlea is in all other respects normal.
150	13	3	130	256 ~	Reissner's and the tectorial membrane are normal. The cells and framework of Corti's organ are everywhere normal apart from the absence of nerve fibrils crossing Corti's tunnel. Gross and widespread degeneration has occurred in the neural elements. The number of the medullated fibres in the modiolus and osseous spiral lamina is greatly reduced. A few cells of the spiral ganglion are still to be seen towards the apex. The spaces of Rosenthal's canal are otherwise empty apart from neurolemmal remnants. There is fine scattered exudate occurring in the perilymph channels indicating recent labyrinthitis, in particular in the scala tympanum of the basal whorl.	Exudate in very small amounts occurs in the scala tympanum of the basal whorl. The cochlea is in all other respects normal.
			110	512 ~		
			90	1024 ~		
			180	2048 ~		

TABLE III

No of animal	Survival time	Wever and Bray response microvolts (Peak)		Histological findings	
		Right ear		Left ear	Right ear
		Weeks	Days		
131	3	760	256 ~	290	
		620	512 ~	620	
		840	1024 ~	700	
		1120	2048 ~	840	
129	1 1	1400	256 ~	Not tested	
		840	512 ~		
		900	1024 ~		
		1120	2048 ~		
125	1 2	920	256 ~	810	
		1040	512 ~	550	
		1060	1024 ~	540	
		1100	2048 ~	100	

There is a small amount of exudate in the scala tympani of the basal whorl. The cochlea is in all other respects normal

Reissner's and the tectorial membrane are normal. Corti's organ presents an appearance in all respects normal. The neural elements show early but conspicuous changes. These are seen chiefly in the cells of the spiral ganglion, in which much cloudy swelling and fragmentation of the nuclei have occurred. No obvious decrease has occurred in the number of the medullated fibres in the modiolus and osseous spiral lamina, and in most of the whorls the fibrils crossing Corti's tunnel are still to be observed. There is a very considerable degree of serous labyrinthitis. A fairly dense exudate is found throughout all the perilymph channels.

The tectorial and Reissner's membrane are normal. Corti's organ is everywhere of normal appearance apart from the absence of nerve fibrils crossing the tunnel of Corti. Early degenerative changes have occurred in the neural elements, though little if any decrease is to be noted in the number of medullated fibres in the modiolus. Cloudy swelling and nuclear fragmentation are well marked in the cells of the spiral ganglion, in the number of which there is a noticeable reduction. There is considerable and widespread labyrinthitis as indicated by the presence of much exudate in the perilymph channels.

Reissner's and the tectorial membrane are normal. No nerve fibrils are to be seen crossing Corti's tunnel. No other departure from normal can be observed in Corti's organ. The neural elements display fairly advanced changes. There is a marked decrease in the number of the medullated fibres in the modiolus and the osseous spiral lamina. The cells of the spiral ganglion are greatly reduced in number and show advanced degenerative changes. A well marked serous labyrinthitis has occurred with scattered exudate in all the perilymph channels.

The perilymph spaces contain scattered exudate and granules in small quantities indicating recent serous labyrinthitis of slight degree. The cochlea is in all other respects normal.

Pigment granules and a very small amount of exudate occur in the perilymph spaces in the basal whorls indicating recent slight serous labyrinthitis. The cochlea is in all other respects normal.

The evidence provided by the findings in these two groups of cases would seem to indicate that following the operation of nerve section, a basal meningitis occurs which is more marked upon the side of operation

A serous labyrinthitis of corresponding severity is set up in the cochlea on each side by direct spread along the cochlear aqueduct. As is well known this provides the sole channel of communication between the subarachnoid space of the posterior fossa and the perilymph spaces of the internal ear and opens into the cochlea in the scala tympani adjoining the round window. In the later stages, resolution of the inflammatory reaction occurs, as shown by the disappearance of serous exudate in any large amount in the perilymph channels. The only visible signs remaining consist of organized coagula around the opening of the cochlear aqueduct, changes which, it is suggested, may be associated with some degree of chronic obstruction to the flow of perilymph. The condition described is represented in fig. 22, Plate 12. The certainty that such obstruction exists cannot of course, be established upon histological data of the kind provided both on account of the physical nature of the coagulum and the obliquity of the plane of section of the aqueduct with respect to its long axis. The presence of this coagulum in the mouth of the aqueduct is, however, clearly demonstrated and the probability is indicated that its functional operation is in the direction suggested.

Striking differences are to be noted in the electrical responses obtained in the two groups of cases. Thus, in the short survival time group with active labyrinthitis, the responses are fully up to normal (0.5–1 mV) and are, moreover, somewhat enhanced upon the side of nerve section in which the serous labyrinthitis is more marked.

In the long survival time group with organized exudate limited to the region of the cochlear aqueduct, the responses are considerably reduced upon both sides, and more so upon the side of operation. The general lines of the findings in these two groups may be considered to support the membrane theory of origin of the cochlear component of the Wever and Bray phenomenon. According to this, the potential changes in question are thought to be engendered by movements of a polarized membrane (Hallpike and Rawdon Smith 1934 *a*). Reasons for regarding the membrane of Reissner as being likely to accord with this description were thought to be furnished firstly by its structure and further by its position separating the perilymph and endolymph. The known differences in origin and composition of these fluids were cited as providing a probable mechanism of polarization. In the cases of the long survival time group now described, the finding of evidence suggestive of chronic obstruction to the perilymph flow provides

upon the basis of the membrane theory a ready enough explanation of a breakdown of the polarization mechanism, which is further correlated with the marked reduction in the electrical response found to occur in these cases. Reference may here be made to a case of this type described in a previous publication (Hallpike and Rawdon-Smith 1934 *b*). In this, absence of electrical response was found to occur in the cochlea of a cat some 12 weeks after VIIIth nerve section. Absence of any morphological abnormality, apart from the absence of neural elements, was then thought to provide support for the neural theory of origin of the Wever and Bray phenomenon.

As indicated in the preceding paper, other evidence is now available which must be considered to exclude the acceptance of this theory. Though, in the case described, sections through the region of the cochlear aqueduct were not available, it seems justifiable to postulate the occurrence of conditions comparable to those demonstrated in the present long survival-time group of cases, and to ascribe to these conditions the absence of electrical response.

In the cases of the short survival-time group, perilymph stasis, probably of acute degree, must be supposed to have occurred. However, the correlation of these findings with the marked preservation of the electrical response would appear to be quite reasonably compatible with the membrane potential theory of origin of the response, if it be supposed that in this acute condition, with gross exudate in all the perilymph channels, the osmotic conditions favouring the maintenance of polarization of Reissner's membrane are preserved.

Grateful acknowledgement is made to the executors of the late Dr A. A. Gray for the loan of photomicrographic apparatus.

#### SUMMARY

The morphological changes which occur in the cochlea following section of the VIIIth nerve are described in a series of eleven cats, and are correlated in each case with the electrical response obtained from the cochlea during acoustic stimulation.

The cases are divided in accordance with the findings into three groups.

##### *Group I*

In this group, marked degenerative changes are found in the neural elements and in the cells of Corti's organ, with preservation of a normal electrical response.



*Group II*

In this group the neural elements show an advanced degree of degeneration. The cells of Corti's organ are however relatively well preserved. Organized exudate is found in small amounts in the scala tympani adjoining the opening of the cochlear aqueduct. The electrical responses are absent or much reduced.

*Group III*

In this group minor degenerative changes are found in the neural elements and cells of Corti's organ. There is a widespread serous labyrinthitis with much exudate in all the perilymph spaces. The electrical responses are normal or enhanced.

The findings in Groups I and II are considered to render untenable not only the neural but also the more generally accepted hair cell theory of origin of the cochlear response.

The findings in Group III and in other cases of the series in which serous labyrinthitis is found to have occurred are considered to provide evidence which supports the membrane theory of origin of the cochlear response.

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 Witmaack K 1911 *Verh dtsch otol Ges* 20 295

## DESCRIPTION OF PLATES

## Plates 7 12

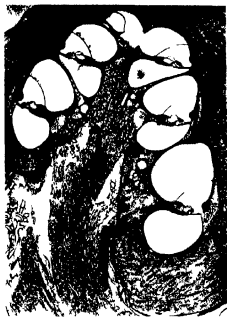
FIG 1—Total necrosis of the contents of the cochlea following section of the VIIIth nerve and cochlear artery. Note the formation of masses of heterotopic new bone.

FIGS 2-9—Photomicrographs of cochleae of Cat No. 147

- FIG 2—Left cochlea × 22  
 FIG 3—Left cochlea Lateral middle whorl × 80  
 FIG 4—Left cochlea Lateral basal whorl × 80  
 FIG 5—Left cochlea Internal apical whorl × 80  
 FIG 6—Left cochlea Internal middle whorl × 80  
 FIG 7—Left cochlea Internal basal whorl × 80  
 FIG 8—Right cochlea × 22  
 FIG 9—Right cochlea Internal middle whorl × 80



*E 1*



*1*



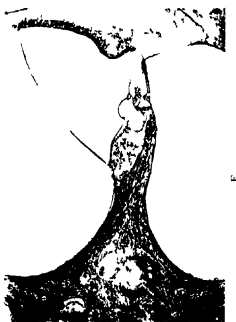
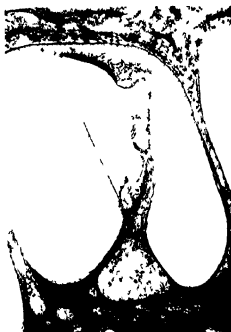




Fig. 9



Fig. 10





Pl. 13



Pl. 14



Pl. 15



Fig. 1



Fig. 11



Fig. 18

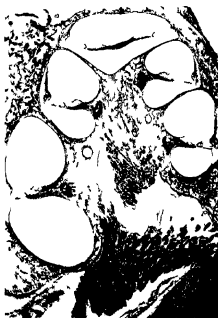


Fig. 2



FIG. 21



FIG. 22

**FIGS. 10-15—Photomicrographs of cochleae of Cat No. 150**

- FIG. 10—Left cochlea.  $\times 22$ .  
 FIG. 11—Left cochlea. Internal apical whorl.  $\times 80$   
 FIG. 12—Left cochlea. Internal middle whorl.  $\times 80$   
 FIG. 13—Left cochlea. Internal basal whorl.  $\times 80$   
 FIG. 14—Right cochlea.  $\times 22$   
 FIG. 15—Right cochlea. Lateral basal whorl.  $\times 80$

**FIGS. 16-21—Photomicrographs of cochleae of Cat No. 131**

- FIG. 16—Left cochlea.  $\times 22$   
 FIG. 17—Left cochlea. Lateral middle whorl.  $\times 80$   
 FIG. 18—Left cochlea. Lateral basal whorl.  $\times 80$   
 FIG. 19—Left cochlea. Internal apical whorl.  $\times 80$   
 FIG. 20—Right cochlea.  $\times 22$   
 FIG. 21—Right cochlea. Lateral basal whorl.  $\times 80$

FIG. 22—The opening of the aqueduct of the cochlea into the scala tympani, showing the presence of a fine organized coagulum. The denser material appearing within the lumen of the aqueduct consists chiefly of its epithelial lining cut in oblique section

612 744 . 15 : 593 . 95

## A Contribution to the Comparative Biochemistry of Muscular and Electrical Tissues

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*(Communicated by Sir Frederick Hopkins, O.M., F.R.S.—Received  
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Recent advances in the biochemistry of muscle have made it clear that the disappearance of glycogen and the resynthesis of phosphocreatine during anaerobic contraction are connected by a complex cyclical system of reactions in which a leading part is played by adenylic compounds (see for example Ostern, Baranowski and Reis 1935, Needham and van Heyningen 1935; Lehmann 1935). The subject has recently been summarized by Parnas (1936), full details and references will be found in his review.



The present paper reports the results of some comparative work on the presence of similar systems in some hitherto unexamined tissues. These were, first, the electrical organs of the ray *Torpedo*, which, although extensively modified in the course of their development, arise from pre-muscular tissue in the embryo and may therefore be regarded in some measure as muscular in origin. We also used the striated jaw muscles of a typical echinoid and the smooth muscles of the body wall of a holothurian. In addition we determined the type of phosphagen present in representatives of two other groups of Echinodermata, the Ophiuroidea and the Crinoidea, which have not hitherto been examined. The results on echinoderm muscle have a significant bearing upon the phylogenetic aspects of our knowledge of the distribution of the phosphagens. A preliminary communication has already appeared (Baldwin and Needham 1936).

The following reactions are known to occur in the presence of enzyme-containing extracts prepared from vertebrate muscles, and are the ones which we have studied here.

- (i) Phosphoglyceric acid  $\rightarrow$  phosphopyruvic acid
- (ii) Phosphopyruvic acid + adenylic acid  $\rightarrow$  pyruvic acid + adenylypyrophosphate
- (iii) Adenylypyrophosphate + creatine  $\rightleftharpoons$  creatine phosphoric acid + adenylic acid
- (iv) Adenylypyrophosphate  $\rightarrow$  adenylic acid + phosphoric acid

Lehmann (1935) and Lohmann (1936) have shown that with invertebrate muscle, as represented by crab and octopus, creatine is replaced by arginine in reaction (iii); it is important to notice that vertebrate muscle will not esterify arginine, nor will the invertebrate muscles previously tested (crab) esterify creatine (Lehmann 1935).

We were able to demonstrate the occurrence of this series of reactions in the electrical cells of *Torpedo* and in the echinoderm muscles studied, the fact that the adenylypyrophosphate used in our experiments was prepared from rabbit muscle strengthens the opinion that one and the same nucleotide is involved as a phosphate carrier in all kinds of muscle, vertebrate and invertebrate alike.

#### METHODS

The tissue extracts used were prepared in some cases by grinding the tissue with sand and adding one and a half times its weight of ice-cold distilled water, in others by freezing the material in a mortar till quite hard,

beating it with the pestle until a finely divided paste was obtained, and adding the water to this. In either case the mass was allowed to stand at 0° C. for 30 min. and then centrifuged, and the extract decanted into a flask to be stored in the refrigerator. Where dialysed extracts were used, the dialysis was carried out in thin cellophane membranes (previously soaked in N/10 HCl for several hours and then well washed with distilled water) against ice-cold 0.5% KCl in the refrigerator, with frequent stirring and changing of the external solution.

The solutions used were the following

*Creatine*, ca. 30 mg./c.c.

*Arginine*, ca. 50 mg./c.c. (approx. equivalent to the creatine)

*3-phosphoglyceric acid*, 2.4 mg. P/c.c., from the barium salt of a preparation made from yeast, barium being removed by a very slight excess of sodium sulphate.

*Adenylpyrophosphate*, 1.4 mg. pyrophosphate P/c.c., from the barium salt of a rabbit muscle preparation, barium being removed as above.

*Adenylic acid*, 0.7 mg. P/c.c., equivalent to the adenylpyrophosphate, from a pure preparation (by Henning) from mammalian muscle.

*Magnesium sulphate*, half saturated, containing about 0.6 mg. Mg/drop.

All solutions were brought to pH 7 by the addition of acid or alkali, phenol red being used as external indicator. Fresh samples were made up at least once a week and often more frequently, and were kept at 0° C.

The following description of a typical experiment gives details of the procedure followed in most of the experiments, and only minor modifications were introduced from time to time where necessary in particular cases. The samples were set up in numbered tubes, each of which received 2 c.c. of the enzyme preparation. After the required quantities of the various reagents had been added, the pH was brought to 7.8–8.0 by the addition of a few drops of 4% NaHCO<sub>3</sub>, phenol red being used externally as indicator. The final volume was 2.9–3.0 c.c. The tubes were now incubated for the required time at the temperature desired, usually 10 min. at 40° C., and at the end of the incubation they were plunged into a freezing mixture to bring the temperature rapidly down to zero. An equal volume of ice-cold 8–10% trichloroacetic acid was now added to precipitate the proteins and inactivate the enzymes. After the addition of 1 c.c. of 25% barium acetate, a drop or two of phenolphthalein was added, and followed by 2N soda, drop by drop, to make the whole fluid just alkaline to the indicator. After standing for 5–10 min. in ice the tubes were centrifuged and phosphagen was estimated in the centrifugates.

We frequently found that traces of inorganic phosphate were carried over into the centrifugates, and accordingly made it our practice to filter these before proceeding to carry out the estimations. All phosphate estimations were carried out by the method of Fiske and Subbarow (1929).

Creatine phosphoric acid was estimated by treating the filtered centrifugates with the Fiske and Subbarow reagents and allowing them to stand for 30 min. before comparing them in the colorimeter. The molybdate used in this method catalyses the hydrolysis of creatine phosphoric acid, which is then complete after 30 min. The method used for arginine phosphoric acid was that of Lohmann (1936) after just discharging the pink colour of the centrifugates with a drop or two of N/10 acid, the fluid was treated with one quarter of its volume of 10% trichloroacetic acid to give a 2% solution, and heated in a boiling water bath for 1 min. After cooling, the colour reagents were added in the usual way and the comparison made after allowing 10 min. for full colour development. This method gives the sum of both forms of phosphagen, from which the amount of arginine phosphoric acid could readily be found by deducting that of creatine phosphoric acid in the few cases where both were present in a solution at the same time.

In order to determine inorganic phosphate and pyrophosphate P we extracted the protein-containing precipitate, which remained after centrifugation of the alkaline extract, with 5 c.c. 0.4N HCl for 5-10 min., and poured off the clear supernatant solution after centrifugation. Provided that enough acid is used for this extraction there is no inherent disadvantage in not removing the precipitated proteins before precipitating the insoluble fraction of the phosphate. The inorganic phosphate was estimated directly on a sample of the solution so obtained, and the pyrophosphate by hydrolysis in N HCl at 100° C. In general, pyrophosphate P can be determined after a 7 min. hydrolysis, but as phosphopyruvic acid was present in some of the experimental samples, having been formed from added phosphoglyceric acid, one sample was hydrolysed for 7 and a second for 60 min. About one-half of the phosphopyruvic acid is hydrolysed in 7 min. so that the pyrophosphate P could readily be calculated. Although only approximate, this method served to give figures of the order of accuracy required here. In this paper the expression "pyrophosphate P" always means the easily hydrolysable P of the adenylypyrophosphate molecule. Estimations of total P were carried out by wet ashing with sulphuric acid and perhydrol after the method of Eggleton and Eggleton (1929).

All figures in the tables are expressed in mg. P in the sample of extract actually used in the particular experiments.

1—EXPERIMENTS WITH THE ELECTRICAL TISSUE OF *TORPEDO*

In spite of the difference in the nature of their respective responses to stimulation, the electrical organs of *Torpedo* and muscle both arise from the same kind of embryonic rudiments. Although very different morphologically, both types of effector show many physiological and pharmacological resemblances, and there is evidence too of a close biochemical relationship between the two tissues. It has long been known that glycogen, creatine, and phosphate are present in the electrical tissue, and Kisch (1930) and later Baldwin (1933 *b*), who also summarized much of the earlier work, both found that the creatine is actually present in the form of creatine phosphoric acid, its physiological behaviour being very similar to that in muscle. Baldwin (1933 *b*) gave some approximate figures for the partition of phosphorus in extracts of the electrical tissue, and the newer figures reported in Table I show how very close is the resemblance between the two tissues in this respect. The data for rat muscle are taken from Cori and Cori (1932) and those for the frog from Eggleton (see Hill and Kupalov 1930).

TABLE I—DISTRIBUTION OF PHOSPHORUS IN ELECTRICAL AND MUSCULAR TISSUE

	Electrical organ <i>Torpedo</i>	Muscle Frog	Muscle Rat
P as mg/100 g tissue			
Inorganic + phosphagen P	60	83	115
Pyrophosphate P	22	30	40
Adenylic acid P	11	15	20
Ester P	5	5	8
Total P	98	133	183
P as % of total P			
Inorganic + phosphagen P	61	62	62
Pyrophosphate P	22	22	22
Adenylic acid P	11	11	11
Ester P	5	4	5

The species employed throughout our work was *T. marmorata*, of which two large female specimens, about 30 cm in diameter, were used. The extracts were almost colourless, slightly opalescent, and quite mobile even at 0° C.

In previous work demonstrating in muscle the occurrence of the series of reactions mentioned above, the difficulty has always been encountered that the adenylypyrophosphatase (the enzyme system responsible for the catalysis of reaction (1v)) is very active and stable. In its presence, the

demonstration of any synthesis of adenylypyrophosphate or phosphagen is difficult, reaction (iii) is reversible, the direction being influenced by the relative concentrations of the reactants. When adenylypyrophosphatase is present, the concentration of adenylic acid is increased and this brings about the breakdown of much of the phosphagen which has been synthesized in reaction (iii). This difficulty has been partly or completely overcome by dialysing the extracts for 12–16 hours at 0° C (Lohmann 1934) or allowing them to stand for 5–10 days at 0° C with a much shorter dialysis period (Lehmann 1935). During such treatment the adenylypyrophosphatase is destroyed or greatly weakened, while the other enzymes are but little affected. In the present work, each of the tissues examined showed considerable adenylypyrophosphatase activity, and Lehmann's method of inactivation was chiefly used, though not always very successfully as the time at our disposal was too short. With dialysed extracts a magnesium salt was added, as Lohmann (1931) has shown the magnesium ion to be a necessary activator for such extracts.

*Transfer of Phosphate from Phosphoglyceric Acid to Adenylic Acid*

*Exp 1*—The extract was dialysed for 7 hours and stood 5 days at 0° C. 2 c.c. were incubated for 2 min. at 40° C with 1.2 mg. phosphoglyceric acid P, 0.14 mg. adenylic acid P, and 0.6 mg. Mg (sample I). As control, 2 c.c. of extract were incubated with water and Mg alone (sample II)—see Table II. This experiment shows the synthesis of adenylypyrophosphate when phosphoglyceric acid is added. Only about 15% of the adenylic acid added appeared in the phosphorylated form, the increase in *o*-phosphate in sample I indicates the persistence of adenylypyrophosphatase, for other experiments show that free phosphate is never formed from phosphoglyceric acid alone.

TABLE II

	Inorganic P	Pyrophosphate P
I	0.112	0.04
II	0.077	0.00

*Transfer of Phosphate from Adenylypyrophosphate to Creatine*

*Exp 2*—The extract was 7 days old and had been dialysed for 7 hours. 2 c.c. were incubated for 30 min. at 40° C with creatine (30 mg.), adenylypyrophosphate (0.936 mg. pyrophosphate P) and 0.6 mg. Mg (sample I). A control (sample II) contained all the constituents except adenylypyrophosphate—see Table III. Here 63% of the added adenylypyrophosphate

remained unchanged, 10% was transferred to creatine and 22% appeared as inorganic phosphate

TABLE III

	Phosphagen P found	Inorganic P found	Pyrophosphate P found
I	0.094	0.203	0.588
II	0	0	0

*Transfer of Phosphate from Phosphoglyceric Acid to Creatine in the Presence of Adenylic Acid*

*Exp 3*—The extract was 3 days old and was undialysed. 2 c.c. samples were incubated at 40° C., each containing 6 mg. creatine and the various constituents shown in Table IV. This experiment shows (a) that phosphate is transferred from phosphoglyceric acid to creatine, for the amount of phosphocreatine formed cannot be accounted for simply by the decrease in adenylypyrophosphate content, (b) that besides phosphagen synthesis, liberation of inorganic phosphate has gone on. This again must have come, in part at any rate, from the phosphoglyceric acid P.

TABLE IV

Sample	mg phos- phoglyceric P added	mg pyro- phosphate P added	Time (min.)	mg phos- phagen P found	mg inorganic P found	mg pyro- phosphate P found
I	1.2	0.28	10	0.25	0.88	0.16
II	1.2	0.28	10	0.24	0.81	0.08
III	1.2	0.28	30	0.17	1.19	0.06
IV	0	0	10	0.00	0.58	0.00
V	0	0	30	0.00	0.56	0.00

That neither phosphagen synthesis nor formation of inorganic phosphate can go on at the expense of phosphoglyceric acid P in the absence of the adenylic compound is shown in the next experiment.

*Exp 4*—An extract 4 days old was used after 7 hours' dialysis. 2 c.c. samples were used in each case and incubated for 10 min. at 40° C. with 6 mg. creatine and the other constituents given in Table V. In sample IV

TABLE V

Sample	mg phos- phoglyceric P added	mg pyro- phosphate P added	mg Mg added	mg phos- phagen P found	mg inorganic P found	mg pyro- phosphate P left
I	1.2	0.28	0.6	0.24	0.32	0.06
II	1.2	0.28	0.0	0.10	0.29	0.09
III	1.2	0.00	0.6	0.00	0.10	0.00
IV	0.0	0.28	0.6	0.00	0.19	0.04
V	0.0	0.00	0.6	0.00	0.08	0.00

the residual activity of the adenylypyrophosphatase is well shown. No experiments were made to show the formation of phosphopyruvic acid from phosphoglyceric acid (reaction (i)) but in one experiment, in which a brei was used it was shown that phosphopyruvic acid functions like phosphoglyceric in bringing about the phosphorylation of creatine.

*Exp. 5*—The brei was made from tissue which had been kept 4 days at 0° C. this was macerated with half its weight of M/30 phosphate buffer at pH 7 and allowed to stand 90 min. at room temperature before use. 1 c.c. of the brei was taken in each tube together with 7.5 mg. creatine and incubated for 10 min. at 40° C. with the further additions shown in Table VI.

TABLE VI

Sample	mg phos phoglyceric P added	mg phos phopyruvic P added	mg pyro phosphate P added	mg phos phagen P found	mg inorganic P found
I	1.25	0.00	0.28	0.068	0.97
II	1.25	0.00	0.00	0.00	0.75
III	0.00	0.00	0.28	0.00	0.88
IV	0.00	1.25	0.28	0.048	1.11

The formation of pyruvic acid as an end product of the breakdown of phosphoglyceric acid was shown in the following way. 2 c.c. of an extract which was four days old and had been dialysed for 7 hr. were incubated with 2.5 mg. phosphoglyceric acid, P 0.28 mg. pyro P in the form of adenylypyrophosphate and 6 mg. creatine, the reaction being allowed to continue for 2 hours at room temperature (about 25° C.). After deproteinizing the mixture with an equal volume of 8% trichloroacetic acid and centrifuging 5 c.c. of the centrifugate were treated with 1 c.c. of a saturated solution of 2,4-dinitrophenylhydrazine in 2N HCl. A yellow crystalline precipitate began to form almost at once and precipitation was complete after standing overnight in the refrigerator. The residual centrifugate was neutralized and submitted to the nitroprusside reaction for pyruvic acid, a strongly positive reaction being obtained. A sample of the extract itself was deproteinized and tested in the same way for pyruvic acid, which was found to be absent.

To sum up these results we may say that not only is the acid soluble phosphorus almost identically distributed in muscle and in electric organ, but the enzyme systems concerned in phosphate metabolism in the two tissues show close similarity. The functional differentiation of muscular and electrical tissue is accompanied by obvious morphological differences yet in embryonic origin, chemical constitution, and in the intermediate, energy

providing chemical reactions the two tissues are closely related. How do they transmute their chemical energy into its characteristic effector forms? Various lines of evidence, in particular X ray studies, have in recent years gone far to convince us that the contractility of muscle is a distinctively molecular contractility and one in which the protein myosin is concerned. The solution of the corresponding problem for the electrical organs seems far distant but perhaps it is logical to expect that here also the characteristic discharge will prove to be associated with the protein architecture of the organ and with the actions of ions upon it.

In conclusion it ought perhaps to be mentioned that the electric organs of *Torpedo* are morphologically similar to those of other fishes such as *Malapterurus* although the latter are derived from glandular rather than muscular rudiments in the embryo and therefore offer a most interesting material for further comparative work upon the lines followed in the present paper.

## 2—EXPERIMENTS ON ECHINOID JAW MUSCLE

The presence in the jaw muscles of *Strongylocentrotus lividus* of both creatine phosphoric and arginine phosphoric acids was demonstrated by Needham. Needham, Baldwin and Yudkin (1932). The only other tissue in which both phosphagens were found was that of an enteropneust *Balanoglossus salmoneus* and these facts led us to suggest that the theory of vertebrate ancestry put forward by Bateson and supported by Macbride and others might claim confirmation of a biochemical as opposed to a purely morphological kind. Needham and Needham (1932) have reviewed other theories of vertebrate descent in relation to biochemical data else where. It will be remembered that the classical work of Bateson led to the belief that the invertebrate group most closely allied to the ancestors of the Vertebrata was the Echinodermata. The Enteropneusta link these two groups since they possess larval forms (*Tornaria*) similar to the larvae of echinoderms and in the adult state a notochord and certain other morphological features characteristic of the Vertebrata.

We had two objectives in the present experiments. It has already been mentioned that in the cases previously studied muscle tissue synthesizes from added guanidine bases only the type of phosphagen which it normally possesses, other bases being unaffected. Thus the synthesis of both phosphagens from added creatine and arginine respectively would be additional evidence for the presence of both in the tissue. Secondly although in the work of Needham and others (1932) the identity of creatine phosphoric acid was



well established less attention was paid to the supposed arginine phosphate. In the present work we aimed at obtaining evidence to prove the identity of the compound synthesized from added arginine with arginine phosphoric acid itself.

Our first experiments were carried out on the jaw muscle of specimens of *Paracentrotus lividus* a species which is abundant at Tamaris but the jaws of which are small for most of the work we used the larger species *Sphaerechinus granularis* from a single specimen of which as much as 1 g of jaw muscle can usually be obtained. We found no reason to believe that there is any significant difference between this and the smaller species at any rate so far as the systems which we studied are concerned. The extracts were prepared in the usual way and stored in the refrigerator. They were very viscous even at room temperature and at 0° C were semi solid. The general experimental method and the solutions employed were the same as for the electrical organs of *Torpedo*.

#### *Synthesis of Creatine and Arginine Phosphates*

We first demonstrated the synthesis at the same time and by the same sample of extract of both phosphagens. It was found that the phosphagens can be synthesized independently or simultaneously but that arginine phosphoric acid is always synthesized more readily than the creatine compound; this is interesting in view of the fact that the results of Needham and others (1932) suggest that the creatine phosphagen is the more labile of the two *in vivo*.

*Exp 6*—In this preliminary experiment 2 c.c. of an undialysed 8 days old extract were used with 6 mg creatine and 10 mg arginine. 1.2 mg phosphoglyceric acid P and 0.28 mg adenylypyrophosphate P were added and the tubes incubated for 10 min at 40° C. when 0.015 mg creatine phosphate P and 0.052 mg arginine phosphate P were found.

In later experiments much better yields of both phosphagens were obtained.

*Exp 7*—An undialysed extract 8 days old was used. 2 c.c. samples were incubated in each tube with the additions given in Table VII for 10 min at 40° C. This experiment indicates that the two phosphagens are synthesized quite independently of each other. There is no evidence to show whether two separate specific enzymes are concerned or whether when one tissue can deal with both bases a less specific enzyme is present than that which occurs in vertebrate or crab muscle.

With an 8 days old extract dialysed for 11 hours all conditions being otherwise the same as in the last experiment a yield of 0.33 mg arginine

phosphoric acid P was obtained (corresponding to 26% of the added phosphoglyceric acid P) while with added creatine the yield of creatine phosphoric acid P was 0.15 mg (corresponding to 12% of the phosphoglyceric acid)

TABLE VII

Sample	mg phosphoglyceric P added	mg pyrophosphate P added	mg adenylic P added	mg arginine phosphoric P synthesized
(a) Synthesis of arginine phosphoric acid (10 mg arginine added)				
I	1.2	0.28	—	0.296
II	1.2	—	0.14	0.176
III	1.2	—	—	0.00
IV	—	0.28	—	Trace
V	—	—	—	0.00
(b) Synthesis of creatine phosphoric acid (6 mg creatine added)				
I	1.2	0.28	—	0.100
II	1.2	—	0.14	0.055
III	1.2	—	—	0.00
IV	—	0.28	—	0.00
V	—	—	—	0.00

It is interesting to notice that adenylic acid acts here quite efficiently in the transport of phosphate from phosphoglyceric acid to the guanine base. Estimations of the easily hydrolysable (pyrophosphate) P in tubes (a) II and (b) II (Table VII) showed that 60–70% of the adenylic acid added had been converted into the pyrophosphate. Also in experiments on holothurian muscle to be described below, practically no difference was found in the amount of phosphagen synthesized in the presence of either of these two compounds. This ready phosphorylation of adenylic acid goes on in vertebrate muscle extracts but was found not to occur in the invertebrate muscle extracts previously studied (Lohmann 1935 and Lehmann 1935 for crab muscle; Lohmann 1936 for the muscle of *Octopus*). Lohmann (1935) has shown that breakdown and synthesis of adenylypyrophosphate takes place in two stages, adenosine diphosphate being an intermediate product.

(v) Adenylypyrophosphate + arginine  $\rightleftharpoons$  adenosine diphosphate + arginine phosphoric acid

(vi) Adenosine diphosphate + arginine  $\rightarrow$  adenylic acid + arginine phosphoric acid

In crab muscle reaction (v) is reversible but reaction (vi) is not; that is to say, adenosine diphosphate but not adenylic acid can be phosphorylated by phosphagen. Similarly, phosphopyruvic acid can phosphorylate adenosine diphosphate but adenylic acid very slowly or not at all. In extracts

of *Octopus* muscle also the phosphorylation of adenylic acid by phosphopyruvic acid seems to be a comparatively slow process

The formation of pyruvic acid from phosphoglyceric acid was demonstrated in an experiment in which 2 c c of a 7 days old extract were incubated with 2.5 mg phosphoglyceric acid P 0.28 mg pyrophosphate P (as adenylypyrophosphate) and 6 mg creatine for 2 hours at room temperature After deproteinizing the fluid a positive nitroprusside reaction and the formation of the 2,4-dinitrophenylhydrazone were successfully demonstrated

Attempts were made to demonstrate also the effect of adding magnesium to dialysed extracts but with no great success After 11 hours dialysis the addition of magnesium did not increase the synthesis of either phosphagen and this may be due to the great viscosity of the extracts at the temperature at which the dialysis was carried out It is possible that the mobility of the magnesium ion in such a medium may be small or on the other hand that magnesium was retained within intact cells for the extracts of the jaw muscle presented the appearance of a very thick suspension of tissue substance rather than that of a strong cell free protein solution such as is obtained from many tissues In another experiment a portion of one of the extracts was dialysed for 3 days in all and it was then found that although the synthesis of creatine phosphoric acid was not much affected that of the arginine compound rose from 0.176 to 0.356 mg phosphagen P with the addition of 0.6 mg of magnesium in a typical experiment

The synthesis of two phosphagens by the normal muscle mechanism has thus been demonstrated the one substance being synthesized from creatine and the other from arginine We shall return to a discussion of the phylogenetic aspect of these results in a later section

#### *Identity of the Arginine Phosphoric Acid*

Several samples of crude arginine phosphoric acid were prepared for this part of the work In a typical case this was done by incubating 2 c c of a 2 days old extract with 1.25 mg phosphoglyceric acid P 0.28 mg pyrophosphate P and 10 mg arginine for 10 min at 40°C and pH 7.8 After incubation the fluids were deproteinized with 1 vol of 10% trichloroacetic acid treated with barium acetate in the usual way and made slightly alkaline to phenolphthalein with 2N NaOH After centrifuging down the precipitate the clear centrifugate was treated with 2 vol 95% alcohol (previously neutralized to pH 9) and left for 2-3 hours to allow of complete precipitation of the phosphagen in the form of its barium salt The precipitate was centrifuged down dissolved in a little weak acid and repre-

precipitated with alcohol again centrifuged down and dissolved again in weak acid. The pH was now brought to 7 and a little sodium sulphate solution was added to remove the barium after which the solution was centrifuged again and finally made up to a known small volume.

(i) In one such experiment a preparation was made up to a final volume of 10 c.c. In 2 c.c. the free arginine content was estimated by Weber's modification (1930) of the Sakaguchi reaction. The remaining 8 c.c. were treated with 2 c.c. 10% trichloroacetic acid and heated for 1 min. in a boiling water bath to decompose the phosphagen. Of the resulting fluid 5 c.c. were taken for determination of the phosphate liberated during the hydrolysis, while the remaining 5 c.c. were used for duplicate determinations of the final arginine content. From the observed phosphate content it was calculated that the total arginine content after hydrolysis should be 1.07 mg. allowing for the 0.058 mg. found in the unhydrolysed solution. The amount actually found was 1.32 mg. in all or about 120% of the yield expected. In view of the approximative nature of the Weber method this agreement is satisfactory, and may be taken as an indication that the phosphagen consists of equivalent amounts of phosphoric acid and a base which gives the Sakaguchi reaction. But this reaction is given by a whole series of guanidine derivatives and further confirmation was therefore required.

(ii) The next attempt to identify the base was made with the aid of mammalian arginase which, so far as we know, is almost perfectly specific (see Baldwin 1936). The only substrate known to be attacked apart from arginine itself is the corresponding  $\alpha$  ketonic acid. The arginase employed was prepared by taking 20 g. liver from a freshly killed rabbit, grinding thoroughly with a little sand till the tissue was reduced to a creamy mass and adding 20 c.c. distilled water. After thoroughly mixing the mass was allowed to stand for an hour to extract the enzyme and then centrifuged to remove the sand and unbroken fragments of tissue, the creamy centrifugate being decanted and stored in the refrigerator.

The 'arginine' phosphoric acid was prepared as before, starting with 10 c.c. of the extract, hydrolysed, and made up to a final volume of 5 c.c. of which 0.5 c.c. was taken for phosphorus estimation. The total P content was 0.668 mg. in all. The residual solution was treated with 1 c.c. of the arginase preparation, the pH brought to about 9.5 using the B.D.H. Universal Indicator externally, and then incubated for 3 hours at 38°C. No antiseptic was added. A control was set up at the same time the 'arginine' solution being replaced by water, in order to allow for urea present in the arginase preparation. After incubation the fluids were

deproteinized by boiling and adding enough acetic acid to get maximum precipitation of proteins and filtering a small part of the experimental reaction mixture was however reserved. The manometric method of Krebs and Henseleit (1932) was used to estimate urea in the filtrates the determinations being carried out in duplicate. After correcting for small amounts of urea found in the controls the yield of urea was found to be 1.27 mg in all or 98 % of the 1.29 mg expected from the phosphate determination. The small sample of the experimental solution which had been reserved was deproteinized with an equal volume of 10 % trichloroacetic acid filtered neutralized and tested for arginine by the Weber technique a perfectly negative reaction being obtained.

The base of the supposed arginine phosphoric acid is thus completely hydrolysed by mammalian arginase with the liberation of a quantitative yield of urea.

(iii) A further attempt was made to identify the phosphagen by measuring the velocity of its hydrolysis in N/10 acid with and without the addition of molybdate. The concentration of the latter was 0.25 % in the final reaction mixture. It is known (Meyerhof and Lohmann 1928) that the hydrolysis of arginine phosphoric acid is strongly inhibited by molybdate the retardation factor being of the order of 15–30 times. Lohmann (1936) states that the concentration of molybdate used in this work was approximately 2 % and that the value of the retardation factor depends upon the concentration of molybdate used; this value also depends upon the purity of the phosphagen preparation to some extent. Nevertheless factors of the order of 15–30 have been obtained with only 0.2–0.3 % molybdate by Baldwin and Needham (1933) for the arginine phosphate of blow fly muscle and by Eggleton (1934) for that of the adductor muscle of *Mytilus* while powerful inhibitions were also recorded by Meyerhof (1928) for the phosphagens of several other invertebrates. It seems that the molybdate effect must be considerably more complicated than has been supposed for it was found by Baldwin (1933 c) that the phosphagen of the cephalopod *Eledone* gives retardation factors of only 3–4 with 0.25 % molybdate a fact which led him to suppose that a new phosphagen was perhaps present here. The subsequent work of Lohmann (1936) has shown beyond doubt however that the phosphagen of the cephalopods is arginine phosphoric acid and too great reliance cannot therefore be placed upon the numerical value of the retardation factor as a specific character of the substance.

In the present instance a solution of arginine phosphoric acid prepared by the method outlined above was made decinormal with respect to HCl and incubated with and without the addition of 0.25 % ammonium molyb-

date at 28° C. Samples were withdrawn from time to time and treated with the Fiske and Subbarow reagents with a view to measuring the increase or inorganic phosphate as the hydrolysis proceeded. Actually however it was found that the blue colorations produced were often more than could be accounted for by the whole of the inorganic P known to be present. A similar state of affairs was encountered in our work on fly muscle (Baldwin and Needham 1933) and we showed that this effect is due to the formation of a silico molybdate from traces of silica present in the solutions and extracts. This reaction takes place if silica containing solutions are treated with molybdate in N/10 acid but not in more strongly acid solutions. This silicomolybdate is reduced by the reagent employed in Fiske and Subbarow's method to give a blue compound and thus simulates inorganic phosphate when the estimations are carried out. In the present case it was noticed that samples incubated with molybdate in N/10 acid went bright yellow the coloration being due presumably to the formation of silicomolybdate. In order to find a way of overcoming this difficulty we tried the effect of precipitating the phosphate with calcium in one case and barium in another the phosphate solution having previously been incubated with molybdate in the presence of N/10 HCl and traces of silicate. The precipitates of earthy phosphates were centrifuged down and dissolved in a little dilute acid before adding the colour reagents and in an experiment in which we took 0.1 mg inorganic P in each of three tubes we found the following results on analysis

Direct estimation	0.150 mg P
After precipitation with barium at pH 9	0.140 mg P
After precipitation with calcium at pH 9	0.098 mg P

We accordingly adopted the method of precipitating the phosphate from our samples by neutralizing with soda to pH 9 and adding the calcium reagent of Fiske and Subbarow spinning down the precipitate and dissolving it in dilute acid before adding the colour reagents. We suppose that calcium owes its usefulness here to the fact that barium molybdate is insoluble and carries down barium silicomolybdate with it even if the solubility product of the latter is not exceeded whereas calcium molybdate and silicomolybdate both appear to be soluble in alkaline solutions (pH 9).

On repeating the previous experiment with precautions to prevent interference by silicate we obtained the results shown in the curves of figs 1 and 2. In both cases the curve found in the presence of molybdate was of sigmoid form the inhibition which was marked at first appearing to pass off in the course of time. We can at present suggest no reason

to account for this, but the fact does serve to emphasize the belief that the molybdate effect is complex and deserving of special study

Assuming that both sets of curves are approximately exponential for the first half of the hydrolysis we calculated the following velocity constants

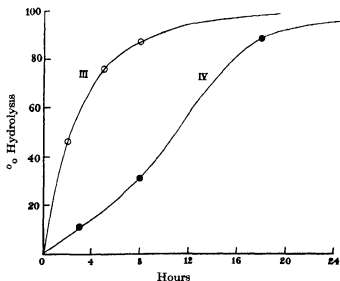
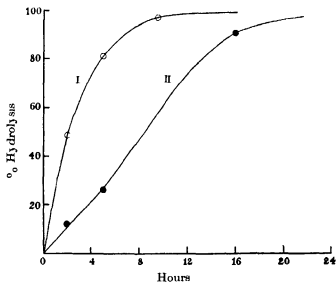


FIG. 1 and 2—Hydrolysis of arginine phosphate synthesized by *Sphaerechinus musculus*, 28° C and N/10 HCl, in presence of 0.25% amm molybdate

which serve to give some kind of numerical value for the retardation factor

	Without molybdate	With molybdate	Retardation factor
Curves I and II	5.5 $10^{-3}$	1.4 $10^{-3}$	4
Curves III and IV	5.3 $10^{-3}$	1.0 $10^{-3}$	5

There is excellent agreement between the two controls, and the value obtained for these agrees well with those in the literature for the hydrolysis of arginine phosphate in N/10 acid (see Baldwin, 1933 a). The small difference between the experiments involving molybdate may be attributed to the fact that the molybdate solution used in the first experiment (curve II) was neutralized to pH 7 before use whereas that used in the second case (curve IV) was a simple aqueous solution, and rather acid. The rate of hydrolysis of arginine phosphoric acid is greatly affected by the hydrogen ion concentration (Meyerhof and Lohmann 1928). The value of 4-5 obtained for the retardation factor is about the same as that found by Baldwin (1933 c) for *Eledone* phosphagen, which, as we have mentioned, must almost certainly be the arginine complex. It is possible that the results obtained in experiments of this kind may depend to some extent upon the sample of molybdate used, the fact that similar results were obtained here and for *Eledone* lends weight to this idea, for the same sample of molybdate was used for both sets of experiments.

Reviewing the evidence for the presence of arginine phosphoric acid in the jaw muscles of the Echinoidea we may say (i) that as regards hydrolysis in acid solutions with and without molybdate, it behaves as would be expected were it arginine phosphoric acid—see Needham and others (1932), (ii) that the compound synthesized by the extract from added arginine is hydrolysed in acid solution at the same rate as arginine phosphoric acid itself, this hydrolysis is considerably retarded by molybdate, the base set free by the hydrolysis gives the Sakaguchi reaction and is rapidly and completely hydrolysed by mammalian arginase with the production of quantitative yields of urea, and (iii) that the presence in the extracts of an enzyme capable of synthesizing arginine phosphoric acid from added arginine is presumably of biological significance, since the tissues of echinoids do contain arginine (Arnold and Luck 1933, Holtz and Thielmann 1924).

### 3—EXPERIMENTS WITH HOLOTHURIAN MUSCLE

The muscles of the Holothuroidea interested us for several reasons. According to Meyerhof (1928) the longitudinal muscles of *Holothuria*



*tubulosa*, a species from which arginine has been isolated by Kutscher and Ackermann(1931), contain arginine phosphoric acid only, while Needham and others (1932) came to the same conclusion with regard to *Synapta inhaerans* Verjbinskaya, Borsuk and Kreps (1935) have studied the phosphagen of *Cucumaria frondosa*, and showed that it behaves in the usual way with respect to various physiological conditions of the muscle itself. They claim, however, that the muscles of this species contain two phosphagens, apparently the creatine and arginine compounds. Unfortunately, we have not been able to see the original paper, which is only known to us through the abstracting journals.

These considerations give added interest to the phylogenetic aspect of the question, and made an examination of holothurian tissue from the viewpoint of its enzyme systems the more important. Furthermore, the muscles of the body wall of the Holothuroidea are unstriated, unlike the echinoid jaw muscles, which are striated, and we welcomed the opportunity of studying a muscle of this type.

Extracts were made from the mixed circular and longitudinal muscles of large specimens of *Holothuria tubulosa*, and experiments were carried out with these preparations in the usual way.

#### *Synthesis of Phosphagen*

*Exp 8*—This experiment was designed to test the possibility that creatine phosphoric and arginine phosphoric acids might both be synthesized by the extracts. 2 c.c. of a 3 days old extract was used in each case with the addition of 1.2 mg. phosphoglyceric acid P and 0.28 mg. pyrophosphate P—see Table VIII. Here again there is evidence for the presence of an active adenylypyrophosphatase, and the synthesis of phosphagen, though small even for the arginine compound, was perfectly definite, and clearly absent in the creatine compound. Larger yields were to be expected in extracts in which the adenylypyrophosphatase had been to some extent inactivated.

TABLE VIII

Tube	Substrate added	mg creatine phosphate synthesized	mg arginine phosphate synthesized	mg inorganic P found
I	6 mg creatine	0.00	0.00	0.33
II	10 mg arginine	0.00	0.04	0.33
III	-	0.00	0.00	0.22

*Exp 9*—Here we used an extract which was 4 days old and had been dialysed for 10 hours. 2 c.c. samples were used in the usual way and

incubated for 10 min at 40° C and pH 7.8. Tube I received 6 mg creatine and the rest, with the exception of tube VIII, 10 mg arginine, 0.6 mg Mg was added to each sample except tube III. The results are given in Table IX.

TABLE IX

Tube	mg phospho glyceric P added	mg pyro phosphate P added	mg adenylic acid P added	mg phosphagen P found
I	1.2	0.28	—	0.000
II	1.2	0.28	—	0.387
III	1.2	0.28	—	0.364
IV	1.2	—	0.14	0.308
V	1.2	—	—	0.000
VI	—	0.28	—	0.012
VII	—	—	—	Trace
VIII	1.2	0.28	—	0.000

Again there was no synthesis of creatine phosphoric acid, although the arginine compound was formed in considerable amounts provided that phosphoglyceric acid was present together with adenylypyrophosphate or adenylic acid. As with sea urchin muscle, the replacement of adenylypyrophosphate by adenylic acid made little difference to the amount of phosphagen synthesized, and we found at the end of the experiment that 0.12 mg of pyrophosphate P had been formed corresponding to the phosphorylation of rather more than 40% of the adenylic acid originally added. No phosphagen at all was synthesized in the presence of phosphoglyceric acid alone, nor in the presence of phosphoglyceric acid and adenylypyrophosphate if arginine itself was absent. A trace of phosphagen was synthesized when only adenylypyrophosphate was added presumably by direct reaction with arginine.

That arginine phosphoric acid alone should be synthesized agrees with the earlier analytical data of Meyerhof (1928) and of Needham and others (1932), but might be regarded as at variance with the results obtained by Verjinskaya and others (1935) on *Cucumaria frondosa*. It would be thought *a priori* that animals so closely related would possess the same phosphagens, but the possibility of species specific differences does, of course, exist.

The present case is interesting from another point of view since so far as we know, these coupled reactions have not previously been studied in unstriated muscle.

We were able to demonstrate the production of pyruvic acid from phosphoglyceric acid in extracts which had been allowed to act on that compound in the presence of adenylypyrophosphate both the 2,4-dinitrophenylhydrazine and the nitroprusside tests giving positive results.

## 4—EXPERIMENTS ON THE DISTRIBUTION OF PHOSPHAGEN

If we leave aside the anomalous result of Verjbinskaya and others (1935) for *C. frondosa*, the data so far obtained for the distribution of phosphagen among the Echinodermata may be summarized by saying that all three of the classes so far examined (Asteroidea, Holothuroidea, Echinoidea) contain arginine phosphoric acid but only the Echinoidea contain creatine phosphoric acid. Reviews of the data have recently been given by Baldwin (1933 *a*) and by Kutscher and Ackermann (1933) and the foregoing experiments confirm these general conclusions. But two important classes of Echinodermata have never been examined till recently, namely the Crinoidea (feather stars) which are believed to be the most primitive echinoderm type and the Ophiuroidea (brittle stars). We are indebted to Mrs P. Baldwin for permission to use her observations on *Ophioderma longicauda* and *Antedon mediterranea* both of which are common near Tamaris. Details of the experiments will be published later. As might perhaps have been expected, *Antedon*, a crinoid, proved to contain arginine phosphoric acid, but no creatine phosphate was found. *Ophioderma*, on the other hand, proved to contain only creatine phosphoric acid, and the arginine compound was not detected. Superficially, the Ophiuroidea resemble the Asteroidea fairly closely, but there is now clear chemical evidence that the two groups are widely different.

TABLE X

Phylum and class	Arginine phosphate	Creatine phosphate
Most invertebrate phyla	+	—
Echinodermata		
Crinoidea	+	—
Asteroidea	+	—
Holothuroidea	+	—
Echinoidea	+	+
Ophiuroidea	—	+
Protochordata		
Tunicata	+	—
Enteropneusta	+	+
Cephalochorda	—	+
Vertebrata all classes	—	+

For the sake of convenience the distribution of phosphagen among the Echinodermata is summarized in Table X, together with that in some other forms which are of interest for our argument. According to the theory of Bateson, the Vertebrata descended from ancestors related to the

Echinodermata through some primitive sort of enteropneust, and so far as our knowledge of the distribution of phosphagen goes it offers support of a chemical kind for this point of view. In the present state of our knowledge, it can hardly be affirmed that the Echinodermata were the only invertebrates in which the production of creatine phosphoric acid was possible, but we have now a fair body of evidence to show that it was only among the Echinodermata that this potentiality was ever realized (see Needham and Needham 1932, Baldwin 1933 a). The fact that the Echinoidea and the Enteropneusta are the only living forms known to contain both phosphagens might be thought to suggest that, of all the Echinodermata, the Echinoidea are the most closely related to the Enteropneusta, and therefore to the Vertebrata. But the modern Ophiuroidea contain only creatine phosphoric acid. Are we to suppose that at some time in their history they possessed both phosphagens and that one of these has since been lost, or that the primitive arginine compound has been replaced by the creatine compound in a single mutation? It seems likely on the whole that the former is the more probable of the two alternatives. The Echinoidea, and possibly certain Holothuroidea are the only echinoderms which contain both phosphagens to day but at some time the Ophiuroidea must probably have passed through the same stage.

There is thus no evidence to show from which echinoderm stock the Enteropneusta branched off, but it is perhaps safe to conclude that they must have arisen from either the Echinoidea or the Ophiuroidea, since the other types do not appear to contain creatine phosphoric acid. The Holothuroidea need further study, *Cucumaria frondosa* appears to contain both kinds of phosphagen, but *Holothuria tubulosa* does not synthesize creatine phosphoric acid even when creatine is put at its disposal. The general conclusion that arginine phosphoric acid is the more primitive of the two phosphagens is borne out by our knowledge of the relative distributions of the two compounds, and among the Echinoidea is particularly emphasized by the fact that it appears very early in the developing embryo, creatine phosphoric acid appearing later on. It would be interesting to study the developing embryos of some ophiuroid species in order to see whether evidence might be obtainable for a recapitulation of the past chemical history of this interesting class.

An investigation of the distribution of enzymes capable of phosphorylating adenylic acid might also yield interesting results. Possibly the similarity noticed in this work between extracts of echinoderm and vertebrate muscles is significant in this connexion. Again, vertebrate extracts differ from those

of crab and octopus in containing an active adenylic deaminase (Lohmann 1935, 1936) and work on the distribution of this enzyme should also be undertaken

The authors wish to take this opportunity of thanking Professor H Cardot, the Director of the Tamaris Station, for his generous welcome and interest. They are also indebted to Mrs P Baldwin for invaluable assistance, and to the Rockefeller Foundation for a grant, made through Dr J Needham, which defrayed part of the expenses incurred in carrying out the work.

#### SUMMARY

1 Extracts of the electrical organ of *Torpedo* contain enzymes which can bring about the transfer of phosphate from phosphoglyceric acid to creatine. This is interesting in view of the origin of the electrical organ from pre muscular rudiments in the embryo. The experimental evidence shows that the series of reactions involved is the same as in vertebrate muscle, and that adenylic acid acts as a carrier of phosphate.

2 Extracts of echinoid muscle contain enzymes capable of bringing about the synthesis of both arginine and creatine phosphoric acids by the usual series of reactions. This fact accords with the previous finding of both phosphagens in echinoid muscle.

3 Extracts of the unstriated muscle of a holothurian can synthesize arginine phosphoric acid but not creatine phosphoric acid. Only arginine phosphoric acid is found to be present in this muscle.

4 Extracts of echinoid and holothurian muscle readily bring about the transfer of phosphate from phosphopyruvic acid to adenylic acid. In this they resemble extracts of vertebrate muscles and show a quantitative difference from the extracts of the invertebrate muscles (crab, octopus) hitherto studied. The latter can rapidly phosphorylate adenosine diphosphate but their action on adenylic acid is slow.

5 The investigation of the distribution of phosphagen in the Echinodermata was extended to include the Crinoidea and the Ophiuroidea. In the former only arginine phosphate and in the latter only creatine phosphate, was found. The bearing of this upon the theory of the evolution of the vertebrates from some echinoderm type through some primitive enteropneust type is discussed in the text.

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# The Foveal Light Adaptation Process

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## INTRODUCTION

This paper is the continuation of one written by the author in 1934 in which the results of binocular measurements of adaptation phenomena were reported. In the method of observation used a patch of light seen in the right eye was compared with a second patch observed in the left eye the right eye was then subjected to a period of light adaptation while the left was maintained in a state of constant (dark) adaptation after which the two patches were compared again. The left eye was thus used as a reference standard against which changes induced in the right eye could be measured. The author's trichromatic colorimeter (Wright 1929) was used in the experiments and it was arranged so that the test patch (the right eye) could be illuminated by any selected monochromatic radiation while in the left eye a mixture of the three instrument primaries was viewed and these could be controlled to produce a match both in colour and intensity between the two patches.

A further series of observations has now been made and is reported here. Some of the earlier experiments have been repeated and a great many new observations have been made but in all the work some important improvements in the method of observation have led to a greatly increased accuracy and significance in the results and have added materially to the information that can be obtained by this method of experiment.

After a period of light adaptation the eye recovers rapidly in sensitivity once the adapting radiation has been removed. In many respects the most interesting characteristic is the state of sensitivity of the eye immediately the adaptation has ceased. In the earlier experiments the adaptation was carried out by directing the right eye down an auxiliary telescope and at the end of the allotted time the observer moved his head over to the two observing telescopes of the colorimeter and proceeded to make a series of matches as rapidly as possible to determine the course and rate of the

recovery process. The sensitivity at the termination of the adaptation had then to be found by a process of extrapolation backwards. Sometimes it was possible to record an observation within the first 5 or 10 sec. but the reliability of the measurement was not good. frequently the next observation was not recorded until 40 or 50 sec. had elapsed from the time adaptation ceased. Hence the information available of the course of the first minute of recovery was very meagre and a good deal of uncertainty existed in the extrapolated value for the initial sensitivity and in the shape of the early part of the recovery curve. If reference is made to the earlier paper it can be seen that the observations were more numerous and more consistent in those experiments in which white adaptations were used than

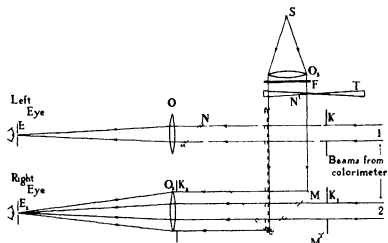


FIG. 1.—Optical system used to view the test patch and the adaptation radiation in the right eye and the mixture of the three colorimeter primaries in the left eye.

in the cases where the eye was subjected to a coloured stimulation. This was probably due to the different methods then employed for obtaining the white and monochromatic adaptations and also to the greater difficulty that is experienced in recording the observations after coloured adaptations due to the fact that changes in both intensity and colour sensitivity have to be compensated during the recovery process.

In order to make more extensive and reliable observations especially for coloured adaptations it was essential to try to devise a method by which the sensitivity immediately at the end of the light adaptation could be determined directly. This was achieved by using the arrangement first employed when determining the effect of adaptation on intensity discrimination (Wright 1935).



The system is illustrated in fig 1, which shows the two beams 1 and 2 as they emerge from the colorimeter the light being brought to a focus at  $E_1$  and  $E_2$  to be observed by the left and right eyes respectively Beam 1 comprises the mixture of the three instrument primaries, while beam 2 is the monochromatic test patch The adapting patch is derived from a pointlight lamp  $S$  after collimation the light is reflected by a mirror  $M$  into the lens  $O_1$  and is finally viewed by the right eye at  $E_2$  When the mirror is in the position  $M$  the right eye sees the lens  $O_2$  filled with light, but if the mirror is rotated to  $M^1$  the adapting light disappears and beam 2 is seen in its place

As the left eye should be in a state of dark adaptation to make an accurate comparison an arm carrying a vertical shutter  $N$  was fixed to the mounting of the mirror When the mirror is in the position  $M$ , the shutter is at  $N$  and obstructs beam 1 When the mirror is rotated to  $M^1$  to reveal beam 2 the shutter moves to  $N^1$  to reveal beam 1 The two beams are thus seen simultaneously and rapidly compared after which the right eye continues its light and the left eye its dark adaptation

With this apparatus it was possible to determine the sensitivity of the right eye in its actual light adapted state before any appreciable recovery had set in If the first comparison was unsatisfactory as it almost always was the adaptation was continued the primaries were adjusted in the appropriate direction and after a period of about 10 sec the beams were compared again This was continued until a satisfactory match between the left and right eyes was obtained It was found that the patches could be viewed for about 1 sec in every 10 sec without affecting the state of adaptation to any noticeable extent

While this method of observation represented the main improvement between the present observations and those obtained earlier, another important modification was the use of a dental fixation to locate the observer's head in a fixed and accurately centred position relative to the exit pupils of the colorimeter Stiles and Crawford (1933) have shown that the apparent brightness of a beam of light striking the retina is very considerably affected by the part of the pupil of the eye through which the beam passes If an accurate comparison between successive experiments is to be made, and if consistent settings in any given observation are to be obtained it is very important that the same part of the pupil should be used throughout the experiments the obvious part of the pupil to choose being, of course, the centre This condition was obtained by embedding a brass plate in Stent's Impression Composition and making a dental impression for the observer The plate was then fixed to the apparatus and

the observer could bite on the plate to fix his head relative to the colorimeter. The adjustments on the exit pupils were used to secure the necessary centering between the eye and instrument pupils.

Due to these alterations it has been possible to make a useful extension to the previous set of observations. The main problem to be attacked has been the extent by which the sensitivity of the eye is depressed as the intensity of the adaptation radiation is varied: the nature of the recovery curves under these circumstances and in particular the variation of these functions when coloured adaptations are used instead of white. The evidence obtained seems to have a very direct bearing on our knowledge of the retinal light sensitive process and on its relation to the triple subdivision responsible for our sensation of colour.

#### EXPERIMENTAL DETAILS

The general character of the experiment was the same as described in the first paper and only a brief description need be given here. For further details the earlier paper should be referred to.

With the exception of the experiment in which the effect of varying the size of the adapting field was investigated the angular subtense of the adaptation patch was about  $5^\circ$ . The two test patches each subtended about  $1 \times 2$ . In all cases the time of adaptation was 3 min: this time has been found sufficient to produce a reasonably steady adaptation level: extension of the period causing no material change in the sensitivity. Before any observations were recorded a period of 30 min dark adaptation was allowed and the left eye was maintained in its state of dark adaptation throughout the series of observations.

Both the initial observation made before the eye was light adapted and that made immediately after the light adaptation to determine the sensitivity level before recovery set in were repeated three times. If the recovery curve was also to be measured it was determined after the third observation had been made. For this purpose the adapting field was swung permanently out of the way and the comparison patches in the two eyes observed continuously. Matches were made as rapidly as possible and the time at which the observation was made as indicated by a stopwatch and the readings on the instrument scales were noted by a second experimenter operating the apparatus. When time permitted the whole experiment was then repeated. Occasionally one set of observations would have to be discarded as being too erratic. Sometimes for example an accidentally high observation might be made and although the observer was imme-

diately aware that it was wrong it might be very difficult to refrain from recording the succeeding points at a similar high level. In the heat of the moment when seconds were precious it might be very difficult to make the conscious effort required to bring the observations down to the proper level.

The readings as first recorded were the scale readings of the colorimeter which give the amounts of the three instrument primaries the red green and blue required in the match. The units in which these were measured were the same as before but are repeated here for reference. The amounts of red ( $r$  wave length  $0.65\mu$ ) and green ( $g$  wave length  $0.53\mu$ ) required in a match of a monochromatic yellow of wave length  $0.5825\mu$  are taken as equal and the amounts of blue ( $b$  wave length  $0.46\mu$ ) and green ( $g$   $0.53\mu$ ) required in a match of  $0.4940\mu$  are similarly adjusted to be equal. This method of obtaining the units and its advantages for visual research have already been described by the author (Wright 1929). The relative brightness of unit quantities of the three primaries for the author's eye are as follows

$$L_r (0.65\mu) = 0.648$$

$$L_g (0.53\mu) = 1.000$$

$$L_b (0.46\mu) = 0.051$$

Any set of observations can thus be transformed into luminosity values if desired. In all cases the results are recorded on the above system but the method only defines the relative magnitudes of  $r$ ,  $g$  and  $b$ . Their actual magnitudes as given in the tables below are arbitrary in the sense that they depend on the filter in the apparatus at the time the calibration curve in use and so on. The absolute retinal intensity of the test colour is also given and is expressed in photons.

The adaptation radiation when derived direct from the pointolite lamp had a colour temperature between  $2800$  and  $2900^\circ\text{K}$ . Its intensity and colour could be controlled by sectors at  $T$  and filters at  $F$  (fig. 1). A daylight filter to raise the colour to about  $4800^\circ\text{K}$  was available and four Ilford spectral filters a red yellow green and blue were used to provide coloured adaptations. The relative energy transmissions of these filters are shown in fig. 2. The adaptations as subsequently specified refer to these filters. The intensities used are also specified in photons.

So far observations have only been made by the author but the results given here represent only a fraction of the total number of his observations since in most cases several sets of confirmatory experiments were made. The general agreement has usually been good although the nature of the experiment is such that an accuracy greater than 10 or 20% can hardly

be expected. There are three possible sources of variation in the results that due to unavoidable errors of observation and experiment that due to unsuspected changes in intensity level in the apparatus and that due to actual changes in the sensitivity of the physiological mechanism from day to day. The first of these was to a large extent countered by repetition of the experiment; the second could be, and to a large extent was either

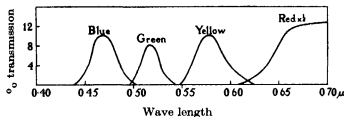


FIG 2—Relative energy transmission of the Ilford spectral filters used in the adapting beam

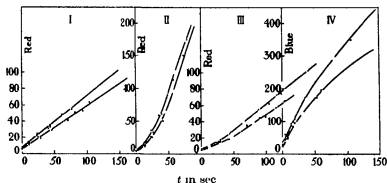


FIG 3—Diagram to illustrate the degree of reproducibility of the results (The dots and crosses represent observations taken on separate occasions several days or weeks apart)

- I Red recovery curve after 2200 photon white adaptation
- II Red recovery curve after 2000 photon red adaptation
- III Red recovery curve after 6000 photon green adaptation
- IV Blue recovery curve after 4800 photon white adaptation

avoided or allowed for, but in a complicated instrument of the kind used it presents considerable difficulty to reproduce exactly the same intensities from day to day, or month to month. In all cases where accurate intensity relations were involved, these were tested in one series of experiments in one observing session. A similar series to test out the same relation would be repeated on another day, and although it might happen that the general level of the results had changed, the same relation among each series of

results would be found to hold. Such measurements also included the effect of the third source of variation, the visual mechanism itself. The value of the results would obviously be greatly impaired if one type of curve were to be found on one day and a different type on some subsequent occasion. This, however, was not the case: the general character of the curves was repeated very satisfactorily as illustrated in fig. 3. Here the recovery curves for four different kinds of experiment are illustrated; in each case two curves are shown corresponding to repeat experiments using similar adaptation and test colour intensities. The implications of the various shapes of curve are discussed in the next section; the point to be noticed here is the similarity in character of the repeat experiments and the appreciable, but not important, difference in actual magnitude of the abscissae.

### RESULTS AND DISCUSSION

*Symbols*—In describing the results the following symbols have been used to indicate the adaptation radiation and test colour:

$A$  retinal intensity of adaptation radiation

$a$  retinal intensity of test colour

$a_t$  apparent retinal intensity of test colour during the course of recovery from the adaptation and at time  $t$  (in seconds) after the termination of adaptation

$r, g, b$  relative magnitude of red, green and blue components of test colour before adaptation

$r_t, g_t, b_t$  relative magnitudes of red, green and blue components of test colour at time  $t$  after the termination of adaptation

The sensitivity of the visual mechanism immediately after the light adaptation has ceased will be indicated by  $a_0$  or by  $r_0, g_0, b_0$  whichever happens to have been measured. The  $r_0, g_0, b_0$  values were generally measured only when an appreciable colour change was induced in the test colour; in the other cases  $a_0$  gives all the information required.

*4800° K adaptation*—The red and green recovery curves obtained with a yellow test colour and various intensities of a 4800° K adaptation are shown in fig. 4.

These curves agree in a very satisfactory manner with those obtained previously. It appears that the linear nature of the recovery does hold in this case from  $t=0$  with the exception of the final red curve which shows the same type of non-linearity found before at high intensities of adaptation. This non-linearity was shown to be due to a radical change in the nature of the visual processes and has been the subject of a further

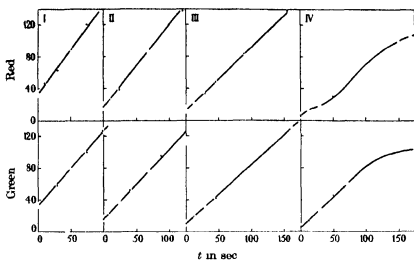


FIG. 4—Red and green recovery curves for  $0.58 \mu$  test colour, intensity 700 photons,  $r=g=220$ , using  $4800^\circ \text{K}$  adaptation. Adaptation intensity in photons: I 1200, II 2400, III 4800, IV 9600.

TABLE I

*Adaptation Radiation—White,  $4800^\circ \text{K}$*

Test colour— $0.58 \mu$ , 700 photons

$A$ (photons)	$a_0$ (photons)	$A/a_0$
1880	105.0	$19.7 \times 10^4$
3760	52.0	$19.5 \times 10^4$
7520	31.4	$23.6 \times 10^4$
15040	16.0	$22.6 \times 10^4$

TABLE II

*Adaptation Radiation—White,  $4800^\circ \text{K}$*

Test colour— $0.58 \mu$ , 250 photons

$A$ (photons)	$a_0$ (photons)	$A/a_0$
1060	39.0	$41.2 \times 10^3$
1510	29.9	$45.2 \times 10^3$
1940	24.6	$47.7 \times 10^3$
2420	19.3	$46.7 \times 10^3$
3240	14.5	$47.0 \times 10^3$
4850	11.5	$56.0 \times 10^3$
8090	4.9	$39.5 \times 10^3$
12900	1.54	$19.9 \times 10^3$

analysis elsewhere (Wright 1936) In the present paper the work has been largely confined to intensities below 10 000 photons

In Table I the values of  $a_0$  for the same experiment and the product  $A a_0$  are tabulated It will be seen that as before this product is constant within the limits of accuracy of the experiment A further set of results for a similar experiment is given in Table II again with the exception of the final value the product is reasonably constant It was shown previously (Wright 1934) that the constancy of this product expresses the fact that the eye tends to adapt itself to a constant response level and it

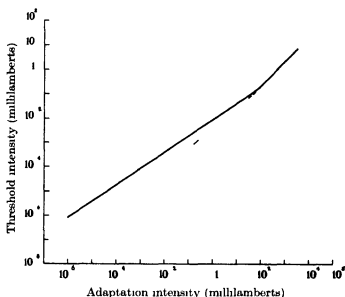


FIG. 5.—Relation between threshold intensity and adaptation level as measured by Nutting (Dotted curve represents a rough attempt by the author to allow for the variation of pupil diameter with adaptation intensity.)

was pointed out that this merely verifies a familiar everyday experience in that for example we are not conscious of any marked difference in intensity level between the artificial illumination of a room at night and the illumination of the same room in the day when we have once become adapted to the particular level. A similar result had in effect been obtained by Nutting (1920). He determined the threshold intensity when the eye was subjected to a very wide range of adaptation levels with the results illustrated in fig. 5. In this diagram the threshold intensity has been plotted against the adaptation intensity using a logarithmic scale for both abscissae. The diagram seems to consist of two straight portions: the upper

part having a slope very close to  $45^\circ$ . On this portion an increase in adaptation intensity necessitates a proportional increase in the threshold intensity the adaptation intensity is thus a constant multiple of the threshold intensity so that assuming the threshold sensation has a constant magnitude the adaptation sensation should also be constant in agreement with our results.

This is very satisfactory confirmation provided the intensity ranges in the two cases are the same. An accurate comparison is impossible because Nutting does not give his results in terms of retinal intensities. If we assume a diameter of 2 mm for his iris at the high adaptation intensities then 1 millilambert gives a retinal intensity of about 30 photons. Our results thus fall on the lower half of the  $45^\circ$  portion of Nutting's curve. Moreover as the pupil diameter varies with the adaptation level the lower adaptation intensities should be increased relative to the higher intensities in the extreme cases by as much as a factor of 10. A rough attempt has been made to allow for the effect of this pupil variation as shown by the dotted curve of fig. 5 and in this curve the proportionality between threshold and adaptation intensities extends over a much wider range. Some deviation is to be expected at low adaptations partly because of the finite magnitude of the threshold and partly because of the incursion of the dark adaptation process which is certainly different from the light adaptation process that we have measured.

The most obvious mechanism by which this constant adaptation level might be produced is that suggested before and supported by the linear recovery curves namely that there is in the retina a reversible photochemical product which is regenerated at a constant velocity. One can hazard a guess that the supply of oxygen to the retina at a constant rate is the controlling factor but this has still to be demonstrated.

The evidence for these conclusions has been derived so far as our results are concerned from the effect of a white adaptation on the red and green responses. A very striking difference is found in the case of the blue response.

Table III gives the  $a_0$  values when using a  $4800^\circ$  K adaptation with a  $0.46\mu$  test colour. It can be seen that the  $A a_0$  product instead of being constant increases in a very definite manner. Also the recovery curves illustrated in fig. 6 are non linear with the exception of the first. Not only are they non linear but their shape is quite different from any of the curves found previously after high intensity adaptations and from the non linear red and green curves that result from coloured adaptations as described later.



It was at first thought that this phenomenon was associated with the positive blue found before (Wright 1934) when using, for example, a yellow adaptation and a yellow test colour. This positive blue was believed to be an after image effect and it seemed likely that the new phenomenon just described had a similar origin. Further tests were first made on the

TABLE III

*Adaptation Radiation—White, 4800° K*

Test colour—0.46 $\mu$ , 30 photons

$A$ (photons)	$a_0$ (photons)	$A/a_0$
390	4.2	$1.64 \times 10^3$
900	1.84	$1.66 \times 10^3$
1200	1.74	$2.09 \times 10^3$
1800	1.66	$2.99 \times 10^3$
3000	1.26	$3.78 \times 10^3$
4800	1.04	$5.00 \times 10^3$
7200	0.77	$5.5 \times 10^3$
14400	0.56	$8.0 \times 10^3$

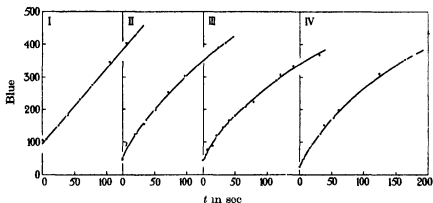


FIG. 6—Blue recovery curves for 0.46 $\mu$  test colour, intensity 10.5 photons,  $b = 720$ , using 4800° K adaptation. Adaptation intensity in photons: I 260, II 600, III 1200, IV 2400.

positive blue to verify that it was a superposed after image. If this were the case the magnitude of the blue should remain unchanged as the intensity of the test colour was varied, if it were due to a genuine alteration in the sensitivity of the blue response, then its value should be directly proportional to the test colour intensity. Results of one such test are given in Table IV. The  $r_0$ ,  $g_0$ , and  $b_0$  values are tabulated when using a yellow adaptation and three intensities of a 0.58 $\mu$  test colour. It will be seen

that the  $b_0$  value remains practically constant as  $a$  is varied whereas the  $r_0$  and  $g_0$  values vary proportionately to  $a$ . We can therefore conclude that this positive blue is an after image effect which while being an interesting and important subject for investigation has not for the present been the subject of any further inquiry.

TABLE IV

*Adaptation Rad at on—Yellow*

Test colour—0.58 $\mu$ 1200 photons $r$ $g$ $b$ 0						
$a$ (photons)	$r_0$	$g_0$	$b_0$	$r_0/a$	$g_0/a$	$b_0/a$
120	16.1	23.4	82.8	0.134	0.195	0.69
240	25.0	40.7	90.0	0.104	0.170	0.375
480	61.5	95.1	96.6	0.127	0.198	0.201

A similar test was then made with a blue (0.46  $\mu$ ) test colour with the results given in Table V. In this case the value of  $a_0$  is roughly proportional to the test colour intensity. There is if anything a slight tendency for the fraction to increase but only to an extent of the same order as the possible observational error. There is certainly no evidence whatever that after images have affected the results when using a blue test colour.

TABLE V

*Adaptation Radiation—Yellow*Test colour—0.46  $\mu$  1200 photons

$a$ (photons)	$a_0$ (photons)	$a_0/a$
2.4	0.114	0.047
4.7	0.282	0.060
7.5	0.396	0.053
15.0	0.816	0.054
30.0	1.87	0.062
60.0	4.07	0.068

One further test was made to determine the cause of the increase in the  $A a_0$  product for the blue. It is shown later that when using say a red adaptation the red component of a yellow test colour is reduced proportionately as the adaptation intensity is increased that is  $A r_0$  is constant but  $A g_0$  increases with the same adaptation. With a green adaptation  $A g_0$  is found to be constant although  $A r_0$  increases. It was therefore thought possible that the increase of  $A b_0$  might be due to the white adaptation not being of a sufficiently blue colour. If  $A a_0$  were only constant for a strictly neutral adaptation it might be that the 4800° K radiation was still somewhat too yellow. This explanation was tested by

repeating the observations using a blue adaptation. The results of this experiment are given in Table VI. Again, the product  $A \cdot b_0$  shows a marked increase as  $A$  increases.

TABLE VI  
*Adaptation Radiation—Blue*  
Test colour—0.46  $\mu$ , 20 photons

$A$ (photons)	$b_0$	$A \cdot b_0$
40	306	$1.22 \times 10^4$
80	136	$1.09 \times 10^4$
150	92.7	$1.39 \times 10^4$
625	71.9	$4.49 \times 10^4$
1500	44.6	$6.7 \times 10^4$

This accumulated evidence points very strongly to the conclusion that the blue receptor and adaptation mechanism is a different and distinct process from those of the red and green. In the previous paper, the results obtained by varying the wave length of the adaptation had led the author to suggest that the blue process was in some way distinctive from the red and green. The facts of colour blindness are also strongly suggestive of the same conclusion. Abnormalities of colour vision are almost entirely confined to the red-yellow-green portion of the spectrum. The occurrence of so-called blue blindness or tritanopia is very rare and its nature doubtful.

If there is this distinctive characteristic about the blue, what is its nature? Some indication is given by the recovery curves of fig. 6, taken in conjunction with the increase of  $A \cdot b_0$ . The rate of recovery as shown by the initial slope of the recovery curves at  $t=0$  is greater in curves II, III, and IV of fig. 6 than it would have been if all the curves had been linear and parallel to I.

Now the amount of decomposition of the hypothetical photochemical substance that will take place in order to produce a balance between decomposition and regeneration will be less the more rapid the rate of recovery, that is, we should expect from curves II, III, and IV that the sensitivity would be reduced to a smaller extent than if the recovery curves had been linear and parallel to I. This is in accordance with the increasing value of  $A \cdot a_0$ . It appears, therefore, that the fundamental distinction between the blue response and the red and green responses is that in the former the rate of recovery is dependent on the concentration of its photochemical material, while in the latter it is independent. This is further borne out by the actual shapes of the curves. The rate of regeneration of a chemical substance is given by

$$\frac{dx}{dt} = k \cdot x^n,$$

where  $n$  is the concentration of the  $n$  products taking part in the reaction and  $k$  is a constant. For a monomolecular reaction  $n = 1$ , for a bimolecular reaction  $n = 2$ , and so on. The curve relating  $x$  and  $t$  is then

$$\int \frac{dx}{x^n} = \int k dt,$$

and since  $x$  is the concentration of the decomposed products of the light sensitive material, the concentration  $Y$  of the reformed material will be

$$Y = X - x,$$

where  $X$  is a constant. The relation between  $Y$  and  $t$  should then correspond to the recovery curves as measured by us. Curves II, III, and IV are of the general type that would be given by the above equations if  $n = 1$  or 2

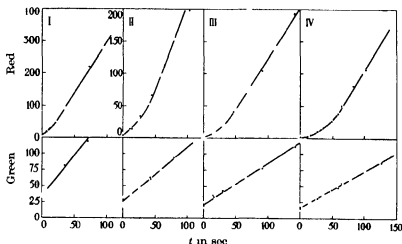


FIG. 7.—Red and green recovery curves for  $0.61 \mu$  test colour, intensity 1100 photons,  $r = 510$ ,  $g = 125$ , using red adaptation. Adaptation intensity in photons: I 900, II 1800, III 3600, IV 7200.

There is thus some evidence for thinking that the blue response could be accounted for by a monomolecular or bimolecular photochemical reaction. The evidence is not conclusive because, for example, the first recovery curve of fig. 6 is linear. On the other hand, there is no evidence at all that the red and green processes ever follow such a course. Here, then, we have some obviously incomplete but very suggestive evidence that may serve to differentiate the blue response from the red and green.

*Red Adaptation*.—In fig. 7 the red and green recovery curves are given as obtained with an orange test colour and various intensities of red adaptation. The green curves are linear as with the  $4800^\circ \text{K}$  adaptation, but the

red recovery shows a very marked curvature becoming more noticeable with increasing adaptation intensities. This curvature had been missed in the previous work owing to the absence of a point at  $t=0$  and to the uncertainty and paucity of the observations during the first 50 or 60 sec.

The first point that should be noted is that the red curves are very different from the blue curves of fig. 6 and there is no possibility of analysing them as a monomolecular or bimolecular reaction, since their curvature is in the wrong direction. The next important point is that the  $A r_0$  values tend to be more or less constant, while the  $A g_0$  product increases with increasing adaptations. This is shown in Table VII, in which the  $r_0$  and  $g_0$  values are tabulated for an experiment similar to that of fig. 7, with a red adaptation and orange test colour. The constancy of the  $A r_0$  values is the more remarkable in that it is the red curves that are non linear, we should rather have anticipated that the linear green curves would have yielded a constant  $A g_0$  product.

TABLE VII

*Adaptation Radiation—Red*Test colour—0.61  $\mu$ , 200 photons  $r=558$   $g=137$ 

$A$ (photons)	$r_0$	$g_0$	$A r_0$	$A g_0$
190	178	150	$3.38 \times 10^4$	$2.85 \times 10^4$
280	152	140	$4.26 \times 10^4$	$3.92 \times 10^4$
390	92.7	109	$3.61 \times 10^4$	$4.25 \times 10^4$
720	36.2	53.5	$2.60 \times 10^4$	$3.85 \times 10^4$
1200	24.3	38.4	$2.91 \times 10^4$	$4.60 \times 10^4$
1800	16.9	30.1	$3.04 \times 10^4$	$5.41 \times 10^4$
2400	14.5	25.5	$3.48 \times 10^4$	$6.12 \times 10^4$
3600	9.1	20.6	$3.28 \times 10^4$	$7.41 \times 10^4$
4800	6.8	16.6	$3.26 \times 10^4$	$7.97 \times 10^4$
7200	5.1	12.7	$3.67 \times 10^4$	$9.15 \times 10^4$

The first deduction that can be made from these results is that the red and green processes are in some way interlocked and associated with one another or with some common visual process. Supposing, for example, that the red and green responses each had their own separate nerve endings in the retina, containing separate photochemical materials, or with the same light sensitive material but different colour filters in each cone, then although a red adaptation would affect the red nerve endings more than the green, the chemical processes would only differ in degree from those occurring with white adaptations. Thus, the recovery curve obtained if the red sensitivity were reduced by white adaptation to, say, one thirtieth of its pre-adaptation value, should be identical with that obtained for the

same sensitivity reduction when produced by red adaptation. The evidence we have is exactly the reverse, so that it appears certain that any theory involving completely separate nerve endings and response paths for the red and green must be to some extent erroneous.

We can see no obvious interpretation of the data at the moment. There may be two processes involved, one responsible for the restoration of the general sensitivity level, some kind of luminosity function, and a second process in series with the first, responsible for restoring the balance between the red and the green sensitivities. Thus the luminosity process might

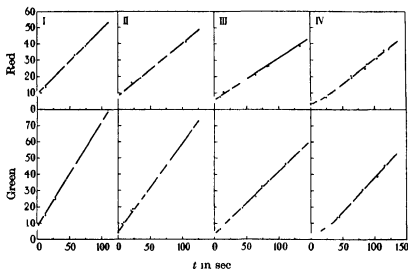


Fig. 8—Red and green recovery curves for  $0.57 \mu$  test colour intensity 220 photons,  $r=70$ ,  $g=130$ , using green adaptation. Adaptation intensity in photons: I 760, II 1520, III 3040, IV 6080.

provide the constant and linear recovery curve, and the restoration of the red-green balance might be responsible for the non-linear curves. But this is certainly an incomplete picture since it does not explain the linearity of the green curves nor the constancy of  $A r_0$  with non-linear red recovery curves and vice versa for the green.

*Green Adaptation*—In fig. 8 the red and green recovery curves are given as obtained with a yellow-green test colour and various intensities of green adaptation. In this case there is less difference from the curves found with  $4800^\circ \text{K}$  adaptation: in fact, a small non-linearity occurs only in curve IV and then in both red and green. On the other hand, the  $r_0$  and  $g_0$  values show a similarity to that found with red adaptations, except that  $A g_0$  is

now the more nearly constant product while  $A r_0$  increases very considerably This is demonstrated in Table VIII which gives the  $r_0$  and  $g_0$  values for a green adaptation and a 0.56  $\mu$  test colour

TABLE VIII

*Adaptation Radiation—Green*

Test colour—0.56  $\mu$  650 photons  $r = 300$   $g = 879$

$A$ (photons)	$r_0$	$g_0$	$A r_0$	$A g_0$
330	122	64.9	$4.02 \times 10^4$	$2.14 \times 10^4$
660	55.3	27.6	$3.65 \times 10^4$	$1.82 \times 10^4$
1220	47.6	21.5	$5.80 \times 10^4$	$2.62 \times 10^4$
2030	31.2	14.7	$6.33 \times 10^4$	$2.98 \times 10^4$
3040	26.9	10.6	$8.18 \times 10^4$	$3.23 \times 10^4$
4050	24.0	8.3	$9.72 \times 10^4$	$3.36 \times 10^4$
6080	17.2	4.9	$10.4 \times 10^4$	$2.97 \times 10^4$
8100	16.4	3.7	$13.3 \times 10^4$	$3.00 \times 10^4$
12200	5.4	2.4	$6.4 \times 10^4$	$2.93 \times 10^4$

The interpretation of the results is not made very much easier by this further information we have the similarity between the red and green adaptations in that the response suffering the greater reduction in sensitivity is reduced in proportion as the adaptation is increased whereas the less depressed response suffers proportionately less reduction as the intensity of adaptation is raised Also the difference in the types of recovery curve with red and green adaptations provides a distinguishing feature between the red and green physiological processes We can claim to have observed characteristics that distinguish all three of the response mechanisms even though we do not know the nature of the physiological processes themselves

*Yellow Adaptation*—In fig. 9 the red and green recovery curves are given as obtained with a yellow test colour and various intensities of a yellow adaptation It is surprising to find that none of the curves is linear They have all been drawn as if consisting of two straight portions the points lie best on a curve of this sort but the accuracy of observation is not sufficiently high to decide for certain whether the points lie on a very shallow curve or on the two lines as shown In the previous work the yellow adaptation had only been tested at 2000 photons and it is easy to see from curve II of fig. 9 how without the initial observation at  $t=0$  the non-linearity would be missed The values of  $r_0$  and  $g_0$  for the same experiment are given in Table IX and the  $A r_0$  product is very accurately constant while the  $A g_0$  values show a slight increase This is probably due to the slightly reddish nature of the adaptation radiation used

The most important conclusion from these results is that there is a difference between the recovery curves for the 4800° K and for the yellow adaptation. This presumably implies some interconnexion between the blue response and the other two responses in the same way that a connexion between the red and green was established above. The recovery of the yellow (if we consider the red and green together instead of separately)

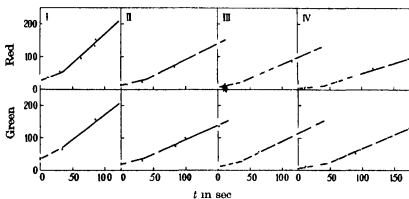


FIG 9—Red and green recovery curves for 0.58  $\mu$  test colour, intensity 700 photons,  $r = g = 230$ , using yellow adaptation. Adaptation intensity in photons: I 900, II 1800, III 3600, IV 7200

TABLE IX

*Adaptation Radiation—Yellow*

Test colour—0.58  $\mu$ , 700 photons  $r = j = 230$

$A$ (photons)	$r_0$	$g_0$	$A r_0$	$A g_0$
900	28.5	36.2	$2.56 \times 10^3$	$3.26 \times 10^3$
1800	14.3	19.5	$2.57 \times 10^3$	$3.51 \times 10^3$
3600	6.9	10.6	$2.48 \times 10^3$	$3.81 \times 10^3$
7200	3.6	5.5	$2.59 \times 10^3$	$3.96 \times 10^3$

is different according as the blue response is considerably depressed (as by the 4800° K radiation) or less depressed (as by the yellow)

**2900° K Adaptation**—Several sets of observations were made with a 2900° K adaptation but the results are not of any unusual interest to need recording here. The recovery curves were in general linear with a tendency in the red curves at high adaptations to follow the shape of those found using yellow adaptations. The  $A r_0$  products were generally constant, while the  $A g_0$  values showed small increases due to the slightly reddish colour of the adaptation.



**Blue Green Test Colour**—Experiments using a blue-green test colour are more difficult than those already described because the test colour lies in a part of the colour triangle that cannot be matched by a positive mixture of the usual instrument primaries. Some experiments have, however, been made by changing the primaries. Thus in fig. 10 the recovery curves are given for a blue green test colour when using a blue adaptation. These curves were obtained by using only two primaries, one at  $0.49\mu$  ( $b-g$ ) and the other at  $0.58\mu$ . It was found that with these two primaries alone a very close match could be made of the appearance of the test colour during

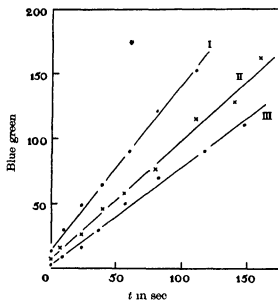


FIG. 10.—Blue green recovery curve for  $0.49\mu$  test colour, intensity 70 photons,  $b-g=220$ , using blue adaptation. Adaptation intensity in photons: I 80, II 190, III 1500.

the course of recovery. Only the blue green curve is given as the amount of yellow introduced was small and rapidly becomes negligible as the intensity of the blue green component increases. It will be seen that the recovery curves are linear. Also, although it was not thought necessary to tabulate these values, in addition to those of Table VI, the  $A a_0$  product increased considerably with the higher adaptation.

In fig. 11 the results found using three different adaptations, again with the blue green test colour, have been plotted. With the green adaptation, blue-green, red, and blue primaries had to be used, but with the yellow and red adaptations, only blue green and blue were required. The blue

green curves indicate the course of the main recovery process and these curves for the green and yellow adaptations show normal linear recovery. In the case of the red the recovery is very much more rapid.

The curves for the other primaries mainly illustrate the recovery of the original quality of the test colour. Although shown on a comparable scale to the blue green they are actually of a much smaller magnitude and less important. The blue curve in II is interesting as it shows some abnormality in the recovery that has not yet been explained. The initial rise of the red in I and the blue in II and III is of interest as showing that the general intensity recovery occurs at a greater rate than the colour recovery.

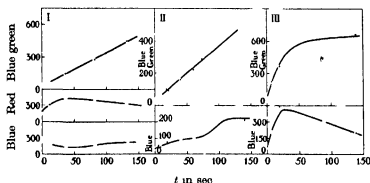


Fig 11—Recovery curves for  $0.49\mu$  test colour intensity 600 photons  $b-g=1000$ . Adaptation radiations I green 4000 photons II yellow 4600 photons III red 3700 photons (The primaries used in recording the recovery are shown on the diagrams.)

Eventually these curves must fall to zero but before doing so they have risen to a maximum.

It should be noted that a similar effect would have been apparent in say fig 7 if the results had been plotted differently. Thus if the data had been recorded in terms of a yellow primary and a green primary as they could have been then the yellow curve would have shown a recovery similar in shape to the red curve of fig 7 while the green would have risen to a maximum and then fallen to zero as in the curves of fig 11.

*Area of Adaptation Field*—A further series of experiments was made to determine the effect of varying the angular subtense of the adaptation field. One portion of the retina is known to affect adjacent portions in certain aspects of vision in particular flicker discrimination ability and contrast. It is of importance to know whether the same applies to adaptation phenomena. A series of five apertures were used to control the area

of the adaptation field and the experiment consisted in measuring the  $a_0$  values for a given test colour and adaptation intensity, when using each of the apertures in turn. It was found that within the errors of the experiment, no difference was apparent. This is illustrated in fig. 12, which gives the  $a_0$  values as measured in three different experiments. In each case the first two observations were discarded as it appeared that these usually gave slightly higher values than subsequent measurements whichever aperture

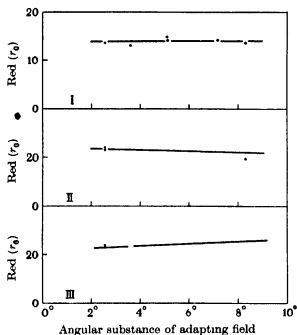


FIG. 12.—Apparent intensity of test colour after adaptation, for varying size of adaptation field. Test colour  $0.65\mu$ , intensity 400 photons. I = 320, II and III = 670, 2900° K. adaptation, intensity 2500 photons. I, II and III represent similar experiments made on different days.

were used first. The apertures were also used in various orders to counteract any possibility of a remanent effect of one aperture upsetting the next observation.

The results show that within the limits tested the area of the adaptation field has no effect on the sensitivity. It seems, therefore, that whatever retinal mechanism is responsible for the process of adaptation, its action is confined to the particular area stimulated, involuntary movements of the eyes probably setting the limit to the extent to which a given state of adaptation can be confined to any one part of the retina.

*Variation of Observer*—One limitation in the work so far described is that all the observations have been confined to one person. At this stage this is inevitable but it will be a decided step forward if the mechanism of even one eye can be elucidated. Nevertheless it is obviously important that similar experiments should be carried out with other observers. A complete range of observations for as many persons as possible would be the ideal but the practical difficulties of time and practice in making the observations are likely to delay the attainment of such a goal. In the meantime a new instrument, a subjective photometer, has been constructed, a first model of which has been described by Wright and Nelson (1935) with this a simple type of adaptation experiment can be performed and the recovery curves for a number of observers using a white adaptation have been measured. These results indicate that about half those tested have adaptation functions similar to the author's; the remainder show considerable variations of one sort and another. It is proposed to confirm and extend these observations with an improved instrument before publication but sufficient data have already been collected to show the importance of making as wide a survey as possible so as to correlate the results with other physiological characteristics of the observer. It is therefore to be hoped that other workers may find it possible to carry out a similar programme of research to accumulate as many data as possible.

*Dark Adaptation and Light Adaptation*—It will have been noticed that in general the recovery curves have not been measured for more than about 3 min. At the end of this time the recovery usually appears to the observer to be complete and any further recovery that may take place does so at a very much reduced rate. Actually about 30 min. have to elapse before the original sensitivity is completely restored. It has been assumed that in the first 3 min. the light adaptation process is at work while in the next 30 min. a second mechanism, the dark adaptation process, is responsible for any developments. It is difficult, however, to justify such a subdivision conclusively although Hecht has produced a formidable array of evidence to demonstrate a clear distinction between the two processes (e.g. Hecht 1935). There is no doubt that after 3 min. all the events of interest have occurred and the characteristics distinguishing one observation from another all appear within this period. No question arises as to whether the whole curve should be measured; it certainly need not be. But it is of interest to know whether the linear recovery curve illustrates the whole course of one process or whether the subsequent flattening out has to be included in a complete analysis.

The total increase in sensitivity during the 30 min subsequent to the first 3 min recovery rarely amounts to a gain by a factor of more than 2 and is frequently less. This represents a very small gain for a dark adaptation effect and illustrates the small extent, if any, of the dark adaptation process occurring at the fovea. Further, it sometimes happens that there is a small colour difference outstanding after 3 min and this may also take 30 min to be completely restored. This certainly suggests that it is the photopic mechanism rather than the scotopic, that is in action. Finally, no physiological evidence for a dual mechanism has ever been demonstrated in the fovea although this may be due merely to the difficulties of investigating minute physiological processes.

It undoubtedly makes the analysis easier if the recovery can be regarded in two parts, but the evidence is on the whole, against the existence of a true scotopic mechanism at the fovea. Possibly the photopic process has some capacity for dark adaptation itself which is different from its normally operating light adaptation process.

*Fundamental Response Curves*—The determination of the fundamental response or sensation curves was attempted in the first of the author's adaptation papers, and a repetition of the experiments with the improved technique to obtain measurements at  $t=0$  and with more observers has not yet been undertaken, but will obviously be useful. We have no reason for doubting the general correctness of the sensation curves from  $0.50\mu$  to the red end of the spectrum although new measurements should result in a considerable increase of accuracy. Some criticism has been made of the curves from  $0.50\mu$  to the violet end particularly in respect of the negative green but no valid reason for rejecting negative quantities has yet been brought to the writer's notice. Nevertheless they are unexpected, and more accurate measurements with the improved apparatus may indicate an error. The additional information we now have regarding blue after images should also be helpful in avoiding misinterpretations.

#### APPLICATIONS

*Theoretical*—The very direct bearing of the results on the theory of the visual processes has already been brought out in the foregoing discussion. It will be apparent that while the three response paths of the Young-Helmholtz theory must exist, there is evidence to support the assumption of a general luminosity process, followed by a subdivision into a blue and, most likely, a yellow process, the latter itself being subdivided into two processes, a red and green. At least there are good reasons to believe that

a linkage exists between the blue and the yellow process and even better reasons for a similar linkage between the red and green. This of course, bears considerable resemblance to certain features of Hering's and other theories.

One important aspect that will have to be considered is the relation between the response measurements obtained by binocular matching methods, and the responses as recorded in the form of retinal action potentials by Granit (e.g. 1933), Hartline (e.g. 1934), Graham (e.g. 1932) and others. Granit and Therman (1935) for example have demonstrated an adaptation phenomena as observed in the case of retinal potentials, which is based on an inhibitory, instead of a photochemical process. The determination of the visual phenomenon corresponding to this process would be of great value.

It will also be important to link up these adaptation measurements with the reactions of the photochemical substances found in the retina, as measured, for example, by Bayliss, Lythgoe and Tansley (1936) and by Chase (1936).

Perhaps the other most fruitful line of development will prove to be the establishment of a relation between the adaptation characteristics and the abnormalities of colour vision. The first step in this direction will be the determination of the adaptation functions of the colour blind themselves. The recovery curves of anomalous trichromats, dichromats, and monochromats should all be of exceptional interest.

*Clinical*—Another use to which the method of experiment, and indirectly the results themselves, may hope to be put is in the determination of the recovery curves of persons suffering from various diseases. This is at present an unexplored field but there are a number of visual and general diseases that must almost certainly affect the adaptation mechanism and the correlation of adaptation data with the other pathological conditions of the observer should be very instructive.

*Illuminating Engineering*—On the more technical side the results can be applied to the proper understanding of the optimum lighting conditions where passage from a well lit scene to a badly illuminated area is in question. For example, the effect on a person passing from a brightly lighted to a poorly lighted street, the time taken to adapt from one level to the other, the most desirable gradation of the illumination under these conditions, are typical problems. The effect of headlight glare and the time required to recover the visual sensitivity is another example. In this connexion in a recent paper by Lythgoe (1936) the question of adaptation from a high level to a low level of illumination was raised and it was stated that

no information was available. The measurements given in this and the previous paper provide data that can be directly applied to this problem. The only other method of obtaining equivalent information is by means of threshold measurements, on the lines of Nutting's work. This is perhaps best regarded as a complementary rather than an alternative method for, while the binocular method provides a more definite form of observation, gives data not provided by the threshold method, and is associated only with the photopic mechanism, yet the latter can be carried out under conditions not possible in the binocular experiments, conditions which may possibly approximate more closely to the practical conditions of the problem.

Reference may also be made to the discussion of a paper by Hopkinson (1936) on the photography of street lighting installations, as an illustration of the way in which the principles of adaptation phenomena may be applied to give the correct interpretation to the technical problems concerned with lighting installations.

Perhaps the most important result on the technical side is the evidence for different adaptation levels for different coloured radiations. For example, a white and a blue source that are equally bright when compared in intensity in a flicker photometer or by calculation from their energy distributions and the standard luminosity curve, will, after the eye has become adapted to them almost certainly produce different sensations of brightness. Thus, for example, a blue and a white that are equal at low intensities will no longer appear equal at high intensities after the eye has become adapted to them. It would thus appear that what may be called the instantaneous intensity, as measured by normal methods of heterochromatic photometry, is not by itself an adequate measure of the visual efficiency of a source, an 'adaptation factor' must also be determined, if a knowledge of the apparent brightness when the eye has become adapted to its surroundings is required.

I should like to express my appreciation of the valuable assistance rendered by Mr J. H. Nelson during the course of the experiments. The co-operation of the person recording the observations is essential if the recovery curves are to be measured successfully. I must also acknowledge with gratitude the continued financial assistance provided by the Medical Research Council.

#### SUMMARY

A further series of observations on adaptation phenomena have been recorded by the binocular matching method, in which effects induced by

light adapting the right eye are measured relative to the constantly dark adapted left eye. An improved method of observation allows the sensitivity immediately at the end of the light adaptation to be determined directly. For white adaptations the sensitivity of the red and green responses is inversely proportional to the adaptation intensity and the recovery curves for these responses are linear for the blue the response is reduced to a less extent than for the red and green and the recovery curves are non linear. They may indicate a monomolecular or bimolecular reaction whereas the red and green processes appear to recover at a constant rate. With red adaptations the green recovery is linear and the red non linear but while the red sensitivity is inversely proportional to the adaptation intensity the green is reduced to a relatively less extent as the adaptation is increased. With green adaptations the reverse occurs. Some non linearity is produced in the red and green recovery curves when yellow adaptations are used. Measurements with a blue green test colour have been made but the practical difficulties of matching with three primaries make a thorough examination in this part of the spectrum awkward.

While still requiring the three response paths of the Young Helmholtz theory the results suggest that there may be a general luminosity process followed by linkages between the yellow and blue responses and between the red and green features strongly akin to Hering's and other theories. The application of the method of observation for clinical purposes is suggested and the relation of the results to illuminating engineering problems emphasized in particular an adaptation factor should be measured in heterochromatic photometry if the true visual efficiency of light sources is to be found.

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## Modification of Mammalian Sexual Cycles, VII—Fertile Matings of Raccoons in December instead of February induced by increasing daily periods of Light\*

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### INTRODUCTION

Since Bissonnette (1932, 1933, 1935*a, b*) discovered that the sexual cycles of ferrets could be altered at will by modifying the seasonal light cycles to which they were exposed, and Baker and Ranson (1932*a, b*, 1933) found that field mice were susceptible to similar changes by manipulation of both food and light cycles, many other studies of sexual photoperiodicity in mammals have been made. These have shown that different species show differences from each other in this respect. Bissonnette has reviewed the literature on sexual photoperiodicity in both animals and plants (Bissonnette 1935*c*, 1936*a, b*), and his papers may be referred to for a more complete treatment of the subject. Some animals, like guinea pigs and 13 lined ground squirrels, are almost if not quite unaffected by changes in duration of exposure to light, at least within very broad limits. Some, like ferrets, come into sexual activity on increasing daily exposures to light. Others, like sheep and deer, become sexually active on decreasing days. And still others, like hedgehogs and field mice, are caused to change their breeding or sexual cycles by other environmental factors as well as by changes in length of day.

An attempt has been made to test the relation of raccoons, *Procyon lotor* (Linn.), to such alterations of daily duration of light. These experiments were carried out at Shade Swamp Sanctuary, Farmington, Connecticut,

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where the junior author is resident director Mr Earl E Bailey of Trinity College Hartford Connecticut took care of the electrical arrangements

#### MATERIALS AND METHODS

##### *Controls*

Controls consisted of the colony of raccoons of various ages and sexes at the sanctuary numbering over fifty animals These were fed and housed in the manner which is routine at the sanctuary Their behaviour and times of holding up in pseudo hibernation were noted and deviated in no respects from normal

##### *Experimental Animals*

The experimental animals consisted of three pens of one male and two females each Of each pair of females one was an animal of known successful breeding during the previous year and the other was either a female which failed to give any young throughout the previous year or one that gave birth to a still born foetus though sexually mature before the previous breeding season These animals were housed and fed in the same manner as the controls and were in the same enclosure which afforded shade from numerous trees All were subject to the same climatic conditions and temperatures varied from well above freezing to as much as 14-20° below zero F with plenty of snow at some times

Each cage consisted of a box like den with sloping roof and holes to permit the animals to go in and out and a runway with floor about 1½-2 ft above the ground This runway was completely enclosed with strong 1 in wire netting except that the den formed one end of it Wide shallow metal pans covered part of the floor of this enclosure and the pans for feeding were placed upon them This conserved the food

In the front wall of the den facing the wire enclosure and just under the roof was cut a hole of proper size to permit a 60 W incandescent bulb to be placed sidewise in such a position that it lighted both the interior of the den and the outside enclosure whenever it was turned on The controls did not have this light Lights were turned on for 1 hr each night from 10 to 17 October 2 hr per night till 27 October 3 hr till 6 November, 4 hr till 17 November 5 hr till 27 November 6 hr till 7 December 7 hr till 17 December and 8 hr per night thereafter till the animals had to be separated because the females were becoming ugly toward each other and toward the male Lights were turned on and off by a time switch and lighting began at six o'clock each evening so that it was easy to see that all lights were lighted each night and none burned out before attendants left for the night

## OBSERVATIONS

*Controls*

The control animals, as is usual with raccoons, showed decreasing consumption of food in November and December and, when snowy weather came on, finally underwent pseudo hibernation. In this, while they were sometimes more or less active within the den, they did not come out for food. Periods of hibernation began at various times with different animals during December and January. The usual routine of decreasing food gradually, to help induce this condition, was followed and the animals ate less and less till they finally failed to come out for any food.

From the first week of February onward, animals of both sexes left the dens and resumed feeding. At these times the first matings occurred and, for individual pens of two females and one male, continued for one or two weeks and stopped.

The times at which different individuals mated varied from this first time in early February to some time in April. In this colony matings under normal conditions have only very rarely occurred before the first week in February and never before the last days of January.

Many of the animals (but not all), after this mating period, again stopped eating and underwent another period or periods of pseudo hibernation in late February and early March. The times varied with individual animals.

It is believed by breeders that the young are born about 63 days after mating. The first litters from these animals were born on 6 and 7 April, but no others before 15 April when litters began to be more numerous. These first litters would place the time of copulation, from which pregnancy resulted, on 2 and 3 February if the period of gestation is the supposed 63 days. The dates of the first litters in the colony in the preceding season were also 6 and 7 April. So the beginning of the receptive time of oestrus and mating is quite uniform from year to year, irrespective of weather conditions, as 1936 and 1935 were quite different as to temperature and amount of snow in December, January, and February.

No resumption of mating after suckling young or after failure to produce litters at the end of the gestation period occurred among controls, nor is it known to have occurred in previous years in this sanctuary.

Winter fur was shed after the birth of litters or at about the same time in non-pregnant animals, not before parturition as with ferrets and some other animals (Bissonnette 1935*d*). Fur was not used in lining the nests made by the females, beginning about 2 weeks before the end of term. Even females that did not produce young from matings did some nest making.

So it is judged from that and from the change in temperament occurring just before that time and the general heaviness of the abdomen even of those that failed to have litters that the raccoon undergoes some sort of pseudo pregnancy. Since most of these animals are very difficult to handle for close observation and palpation even if fairly tame at other times and many (but not all) become very vicious near the end of term diagnosis of pseudo pregnancy was only made from observations of change of behaviour and nest building in the dens. Also the animals were on loan from the State and we were not permitted to kill them for histological study to confirm the superficial diagnosis of pseudo pregnancy. So this term should be understood in this paper as subject to limitation.

This change of temperament and behaviour of the females near the end of term took the form of general surliness and a disposition to drive the male out of the den and not permit him to re enter and to fight among themselves if more females than one were in the same pen.

#### *Experimental Animals*

The first deviation from normal behaviour in the experimental animals was failure to show the gradual decrease of food consumption in autumn exhibited by the controls. The lighted animals remained ravenously hungry even when food supply was being cut down to induce pseudo hibernation so it had to be increased again to normal amounts. They remained active and showed no signs of going into pseudo hibernation in December when most of the controls holed up and stopped eating.

First signs of mating occurred in one pen on 16 December. The male copulated with both females frequently for about a week and then appeared to stop doing so. Those in the second cage went through the same performance beginning on 19 December and those in the third on 23 December. In all three pens both the good breeder of the previous year and the infertile one mated repeatedly (contrast with the first week of February or later for controls).

During a time of very heavy snow with temperatures hovering between zero F and 6-8 below each night the experimental animals remained in their dens for three or four days between 20 January and 10 February. This was the nearest approach to pseudo hibernation shown by the experimental animals and even then they were active within the dens.

Before 28 January the females in the first pen to mate became very cranky and drove the male out of the den and would not let him re enter. So he had to be removed to another pen. On 30 January the two females in this pen had to be separated because of fighting and the lighting was

discontinued with one of them. The members of the other pens had to be separated for the same reason on 11 February.

On this date it was also noted that the females first separated had built nests for the reception of young. So on the basis of their behaviour it was judged that these females were pregnant. Their mammary glands were also enlarged and their abdomens distended. Some of them were very ugly and would claw the attendant at every opportunity. This was not true of any of the controls at this time.

On 27 February a large female from the first pen to begin mating gave birth to four young. She had her experimental lighting stopped on 30 January. On 4 March a female from the second pen to begin mating gave birth to one young animal. On 10 March a female from the third cage to begin mating gave birth to three young. One of these females had reduced lighting from 11 February, the other had not. So continuation of experimental lighting was not a necessary factor in completion of gestation. The other female in each pen failed to give birth to young just as two of them had failed under normal conditions in the previous season. The third had only a still born foetus the previous summer and it was a month later than usual. But all three of these pseudo pregnant females built nests like those of the animals that had litters.

Another striking fact is that those females that did give birth had the same number of young ones under these conditions as under normal ones in the previous season. This would seem to indicate that we were dealing with animals of definitely limited breeding capacity or with none at all. Obviously it is useless to keep as breeding stock females that fail to produce young ones during two seasons or even those producing only one in a season.

On 15 April two of the experimentally lighted females were beginning to shed some hair. None of the others was doing so, however. These were females that failed to have young from the early matings.

"Lighted" males were put back in the cages with the females about 20 March and one of them copulated at once. The other two began to do so frequently from 26 to 29 March. Again they were only pseudo pregnant, but became "ugly" and built nests before the end of term. Two of these females had been receiving added light continuously from the beginning of the experiment, one had not, whereas two that had young had their lighting reduced to normal when they were separated from the males and from each other on 30 January and 11 February. The other had not. This second mating period did not occur among controls nor in other seasons under normal light cycles.

On 15 April the first litter of four young was about ready to be weaned, the others soon afterward. None of the young born in February and early March under these experimental conditions died or was killed by the mother before weaning. They grew and thrived as well or better than those born at normal times, and appeared to reach full size and complete development before 1 October. Further experiments with them are under way to see if they can be induced to breed in their first or second autumns instead of only after 2 years of age as is the case with raccoons produced under normal conditions.

On 18 and 19 May the three females that had weaned their young ones were returned to the lighted cages and to the lighted males. Two of them were seen to copulate again repeatedly on 24 and 25 May and on subsequent days, but not the third, which however, may have done so in the den or at night. No pregnancies ensued. This second mating period did not occur with these females or males in natural conditions during the previous year, nor among the controls in the sanctuary during this season. These second matings and those of the females that failed to have young at the first matings are therefore unusual and may be attributed to the increased exposures to light involved in the experimental treatment of these animals. They mark an increase of breeding activity for this species over that occurring under normal seasonal length of day. This in a sense parallels the increased egg production induced in pheasants under similar night lighting reported elsewhere (Bissonnette and Csech 1936a, b).

In the previous year none of these females mated before 1 February, and the first young born this year among the controls coincided exactly with the first young ones of the previous year. So the births induced by the experimental treatments were just 38 days earlier than the earliest ones this year, and for the first female to have them, just 40 days earlier than in the previous year.

#### (CONCLUSIONS)

It therefore appears that the breeding season of the raccoon may be moved forward from February to December and that young born at least 40 days earlier than normal for a given mother can be reared successfully in the type of pen used at this sanctuary, even through subzero temperatures and heavy snowfall, without artificial heating of any kind and with or without continuing experimental lighting after the pregnant females become "ugly" toward the end of their gestation periods.

The three females rendered only pseudo-pregnant at the light-induced December matings underwent second mating periods before 27 March

when returned to experimentally lighted males, but again they were only pseudo pregnant. They had also failed to have living young or any at all under normal conditions during the previous breeding season. They were therefore apparently useless as breeders, at least with these males. This increase of daily exposure to light did not increase their fecundity, though it did prolong their mating season and induced a second oestrus or heat period beyond the "normal".

Two of the three females that produced young from the December matings were seen to mate again when returned to the males after weaning their young on 18 May. The other may have done so at night or in the den, but was not observed to do it. This also is not found to occur in controls or on normal light cycles. Their failure to become pregnant at the second matings with the males induced to become sexually active in December may perhaps have been due to regression of sexual activity or at least of sperm production in these males after the early stimulation, such as occurs in ferrets and starlings as described by Bissonnette (1933, 1935a, b, 1936a). He has also shown that libido and willingness to mate persist longer than ability to impregnate females in ferrets. Or the result may have been due to lesser intensity of the "oestrus" at this second mating time.

This second mating period per season has not been known to occur in the colony at the sanctuary under normal seasonal light cycles. So it is evidence of increased duration of or recurrence of sexual activity in raccoons subsequent to the light induced earlier mating. Had the males used in this second mating period been on normal light cycles or even on retarded cycles perhaps second litters might have resulted. Development of mammary glands also must go on normally under these conditions, since both animals with which lighting was continued and those with which it was not successfully suckled their young to weaning and the young grew and thrived well from this suckling.

Increase of daily periods of exposure to light in autumn appears to prevent the normal December and January periods of pseudo hibernation or "holing up" in raccoons, but permits short 3-4 day periods of such reduced activity after early February matings. These periods occurred about the time that animals on normal light cycles came out of pseudo hibernation and began to mate, or somewhat earlier. Animals on normal cycles of length of day also showed these periods of pseudo hibernation after matings in February.

The altered light cycle appears to have a less noticeable effect on the hair cycles of raccoons than it does on those of ferrets (Bissonnette 1935d), though this was not carefully watched nor exact dates noted. The normal

shedding of winter coat in raccoons follows parturition instead of preceding it as it does in the ferret. It occurs much longer after early spring oestrus than in the ferret with its later onset of oestrus. Lighted female raccoons, however, shed their winter coat much earlier than those producing and suckling young.

Marked changes in the behaviour of the pregnant and pseudo-pregnant females toward males and toward one another preceded the termination of the gestation or the pseudo pregnant period. This was shown by a general surliness, by ostracism of the males, and by fighting among the females. Such changes were as marked in females subjected to winter gestation periods as in those mating at the usual time from early February onward, and as marked, apparently, in pseudo pregnant as in pregnant animals. Only one of each (pregnant and pseudo pregnant females), however, was in each pen and the fighting may have been initiated mostly or altogether by the pregnant individual. Not all females, however, become surly, either among controls on normal light cycle or among experimental animals.

The oestrous cycles in December, induced by experimentally increased daily periods of exposure to light, and the pregnancies and pseudo pregnancies resulting from matings under such conditions, appear to be normal in every respect but time of occurrence. The three males were activated at the proper time with reference to oestrus of the females and produced sufficient sperms to fertilize the eggs ovulated by the females. Accessory organs and sexual libido were also normal or effective. This is evident, because one female from each pen, and, therefore, with each male, gave birth to one or more young animals.

It is therefore possible to control the time of breeding season of the raccoon. It may be possible to prolong it by controlling and modifying the cycles of duration of daily exposure to light for both sexes, just as with ferrets (Bissonnette 1932, 1935*a, b*, 1936*a, b, c, d*), pheasants (Bissonnette and Csech 1936*a, b*), or bob white (*Colinus virginianus*) (Bissonnette and Csech 1936*c*) which were so produced. Viable young animals have been reared and weaned in the winter season of normal sexual quiescence of raccoons. Under such experimental lighting in winter, the normal period of pseudo hibernation in December and January appears to be omitted and consumption of food does not decrease and stop, as it does in controls or in animals on normal light cycles under similar conditions of temperature.

#### SUMMARY

By increasing the daily periods of illumination from 10 October onward, three male raccoons were induced to become sexually active in December.



and to mate with two females each similarly brought into oestrus in December instead of February

Three of the females were successful in rearing young during the previous season and they again gave the same number of young under the experimental lighting (four one and three respectively) as under normal conditions in the previous year with and without continued lighting after the last 2 weeks of gestation (this in spite of subzero weather) So lactation and maternal instincts were normal

Three other females non breeders in the preceding season again failed to have young under experimental lighting but were only pseudo pregnant

These non pregnant females mated again with the lighted males on 20-29 March after completing their pseudo pregnant periods but again no young were begotten

Two of the three pregnant females returned to the lighted males in May after weaning their young mated a second time the third was not seen to do so No young were produced

Failure of the males to impregnate these females at the second mating periods in March and May was probably due to regression of their spermatogenic functions succeeding their early sexual activity caused by change of lighting or the second oestrus of the females may have been subnormal and may not have been followed by ovulation

The breeding season of the raccoon may therefore be advanced 40 days by increased daily exposure to light in autumn Young ones so produced were successfully reared through early spring months grew as well or better than those born at normal dates and reached maturity of size earlier than usual

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# The Effect of a Glaring Light Source on Extrafoveal Vision

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## INTRODUCTION

When an exposed light source is present in the field of view the visibility of neighbouring objects is impaired owing to what may be called the glare effect of the light source. This glare effect has been studied by several investigators who have measured the increase in the smallest perceptible brightness difference (brightness difference threshold) when the glare source is introduced in such manner that the brightness distribution in the field remains otherwise unchanged. In all the earlier work attention was confined to the case when the subject viewed the brightness difference to be perceived by direct or foveal vision. For this case the investigations of Luckiesh and Holladay (1925) and of Stiles (1929) have shown that if the field has a uniform brightness of  $B$  c/sq ft and if a point source located  $\theta^\circ$  from the test object produces an illumination of  $E$  ft c on the pupillary plane at the subject's eye then the brightness difference threshold  $T$  is raised from the value  $T_0$  appropriate to the field brightness  $B$  to the value  $T_G$  appropriate to a field brightness  $\beta$  where

$$\beta = B + kF/\theta^n \quad (1)$$

Thus the effect of the glare source on the brightness difference threshold is reproduced by superposing a uniform brightness  $\gamma = kF/\theta^n$  on the field brightness  $B$ .  $\beta$  is termed the equivalent uniform field brightness\* and  $\gamma = kF/\theta^n$  the equivalent veiling brightness of the given glare condition. Various values of the constants  $k$  and  $n$  have been obtained by different investigators but the best representative values may be taken as  $n =$  approximately 2,  $k =$  approximately 10. (See Appendix.) It has been shown

\* The term 'equivalent background brightness' has been used in the past but 'equivalent uniform field brightness' has been suggested as more appropriate and we shall adopt this suggestion.

(Stiles and Crawford 1932) that the value of  $\beta$  for a given glare condition is independent of the size colour and exposure time of the brightness difference used in the determination of the difference threshold

More recently the present authors (Stiles and Crawford 1934) have studied the sensitivity of the eye under different brightness distributions including conditions of glare for the case in which the test object is imaged at a point in the parafoveal retina separated  $5^\circ$  (external angle) from the fovea centralis. Owing to the functioning of both rod and cone mechanisms at the  $5^\circ$  parafoveal point the variation of liminal brightness increment\* with field brightness was found to be of a more complex character than for foveal vision. It appeared nevertheless that the effect of a glare source could be reproduced as for foveal vision by superposing an additional uniform brightness over the field. For the particular case of  $\theta = 6^\circ$  it was found that the additional brightness  $\gamma$  equalled  $0.3E$  whereas the foveal formula with  $k = 10$ ,  $n = 2$  gives  $\gamma = kE/\theta^n = 10E/6^2 = 0.28E$ . Thus the equivalent veiling brightness is of the same order of magnitude for foveal and  $5^\circ$  parafoveal vision.

A systematic study was then undertaken to discover whether formula (1) held good for  $5^\circ$  parafoveal vision and also for more remote extrafoveal points. The results of this work are given in the present paper.

It may be noted in connexion with formula (1) that  $E$  represents the illumination on a plane containing the eye pupil. As the glare source is in general located at a certain angle with the direction of vision  $E$  is not equal to the illumination  $E_N$  produced by the glare source at the eye measured on a plane placed normal to the rays from the glare source. For the investigations on foveal vision the angle concerned ( $\theta$ ) has never exceeded  $30^\circ$  and the difference between  $E$  and  $E_N$  can be neglected. For the extrafoveal measurements however larger angles have sometimes been used and it is then necessary to distinguish between  $E$  and  $E_N$ . We shall find it convenient to base our discussion on formula (1) modified by the substitution of  $E_N$  for  $E$ , i.e. on the formula

$$\beta = B + kE_N/\theta^n \quad (2)$$

\* The term liminal brightness increment (l.b.i.) (Stiles and Crawford) represents a somewhat different concept from that described by the term brightness difference threshold (b.d.t.). Where all brightnesses in the field have the same spectral composition as in the present experiments the l.b.i. is defined as the smallest difference of brightness between a given test spot and its immediate surround such that the presence of the test spot can just be detected: the test spot brightness being greater than the surround brightness. For this case l.b.i. and b.d.t. reduce to much the same thing. All the measurements to be described here are strictly measurements of the l.b.i. and we shall therefore use that term.

## GENERAL PLAN OF THE EXPERIMENTS

The position of an object in the visual monocular field is specified in a system of polar coordinates  $r, \phi, \psi$  with the eye as origin and the direction of vision as pole.  $r$  is the distance in feet,  $\phi$  is the angle between the fixation direction and the line joining the eye to the object and  $\psi$  is the azimuthal angle measured from a reference plane which, with the eye upright and looking horizontally, is taken as the horizontal plane on the temporal side.  $\psi$  is measured clockwise from the reference plane for the left eye, anti clockwise for the right eye, so that an object having the coordinates  $r, \phi, \psi$  in the visual field of one eye will coincide with the mirror image in the medial plane of an object with the same coordinates in the visual field of the other eye.

There are four special points in the visual field which have to be considered in the present investigation.

The fixation point  $\phi = 0$

The blind spot centred about the point  $\phi = 17^\circ$  approximately  $\psi = 350^\circ$  approximately

The test spot position  $\phi = \phi_T, \psi = \psi_T$

The glare source position,  $\phi = \phi_G, \psi = \psi_G$

The position of the glare source with respect to the test spot is defined by the angle  $\theta$  between the lines joining glare source and test spot to the eye, and by the angle  $\chi$  which is defined as the amount of rotation about the test spot direction as axis, of a half plane terminated by the test spot direction which in its initial position contains the fixation point and in its final position, the glare source.  $\chi$  is measured clockwise for the left eye, anti clockwise for the right eye. The formulae

$$\left. \begin{aligned} \cos \theta &= \cos \phi_T \cos \phi_G + \sin \phi_T \sin \phi_G \cos(\psi_T - \psi_G), \\ \cos \chi &= \frac{\cos \phi_G}{\sin \theta \sin \phi_T} - \cot \theta \cot \phi_T, \end{aligned} \right\} \quad (3)$$

are obtained by spherical trigonometry.

Fig. 1 shows the relations between the different angles involved. The lines in the figure, shown for ease of drawing as straight, must be thought of as parts of great circles on the unit sphere with the eye as centre. The letters have the following meanings:  $T$  = test spot direction,  $G$  = glare source direction,  $F$  = fixation direction.  $FH$  = horizontal great circle.

In order to test the formula (2) for extrafoveal vision, four sets of experiments were undertaken. It will be noted that the formula does not involve the angle  $\chi$  which determines the orientation of the glare source, with respect to the test spot. Thus the values of  $\beta$  and hence the l b i values  $T$

for a glare source of constant intensity  $E_N$  at the same angular distance  $\theta$  from the test spot but at different orientations  $\chi$ , should all be the same. In one series of measurements, therefore, the glare source was moved round on a circle with the test spot as centre and the l b i was measured with the glare source at various positions on the circle. To test formula (1) with respect to variation of  $\theta$ , it was necessary to determine for each test spot position the curve connecting the l b i value  $T$  with field brightness  $B$  in the absence of the glare source. When this had been done the l b i was

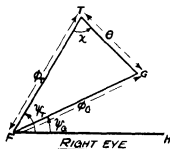
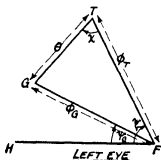


FIG. 1

measured for a glare source of constant intensity  $E_N$  at different angular separations  $\theta$  and the corresponding values of  $\beta$  could be determined from the curve relating  $T$  and  $B$  in the absence of glare. In a third series of measurements  $\theta$  and  $\chi$  were kept constant and the intensity of the glare source was changed. According to the formula the values of the equivalent uniform field brightness determined from these tests should be linearly related to the eye illumination  $E_A$  determining the glare source intensity  $E_N$ . Finally since a given eye illumination  $E_A$  could be produced either by a glare source of high brightness and small angular dimensions or by an extended glare source of lower brightness tests were made to find out whether the l b i and hence the equivalent uniform field brightness  $\beta$  was

the same in these two cases as is indicated by the formula (2)

Throughout the experiments the glare source was presented on a background of zero brightness so that the equivalent uniform field brightness  $\beta$  and the equivalent veiling brightness  $\gamma$  reduce to the same quantity. The test spot used in all the measurements had the following specification

Circular test spot of diameter subtending an angle of  $0.072^\circ$  at the subject's eye, exposed for a period of 0.05 sec. once every 3 sec.

The glare sources, test spot and field brightnesses employed were all of "white" light (radiation from gas filled tungsten filament lamps or pointlights having a colour temperature of about  $2800^\circ \text{K}$ )

## DESCRIPTION OF APPARATUS AND NOTES ON THE METHOD

The apparatus used has already been described as 'threshold apparatus I' in a previous paper (Stiles and Crawford 1934) and it is only necessary to note the special arrangements for the present tests. Referring to fig. 2 the mirrors  $M$  could be adjusted to throw the test spot image on any part of the background screen  $S$  or  $S_1$ . Instead of forming a glare source image in a hole in the background screen it was more convenient to mount the glare source on a fitting inside the cube. Several methods were used for the different experiments in every case care being taken that no light

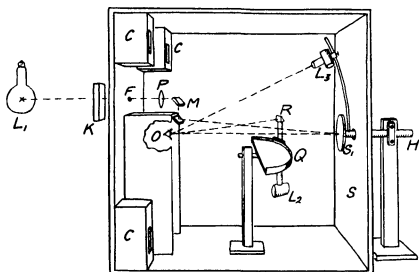


FIG 2

from the glare source fell on the background screen  $S$ . For the measurements with  $\theta$  and  $E$  constant,  $\chi$  varying, the glare source consisted of an aperture with opal glass behind mounted in the side of a metal box  $L_3$  enclosing a 36 watt headlight lamp. The box could be clamped at any desired position on a long rod which was arranged to rotate about a horizontal axle  $H$  pointing at the eye  $O$ . A screening tube projecting out from the box and enclosing the illuminated aperture at one end, confined the rays from the aperture to a limited pencil no larger than necessary to cover comfortably the subject's eye. The test spot was focused at the centre of the subsidiary screen  $S_1$  covering the axle rod. To obtain a variation of  $\theta$  the glare source was enclosed in a metal box  $L_2$  at the lower end of a tube. A right angled prism  $R$  at the top of the tube redirected the beam into the subject's eye.

The tube was made to move round on a curved rail  $Q$  which was adjusted so that the image of the glare source seen by reflexion in the prism described a circular arc with the subject's eye at the centre and lying in a plane containing the test spot image projected on the screen  $S$ . The prism construction was adopted to enable the glare source to be brought as close as possible to the line joining the test spot image to the eye. For the tests on the effect of size and intensity of a glare source at a fixed angular position the glare source suitably enclosed in a light tight box was mounted at a fixed position in the cube and the size varied by means of an iris diaphragm the intensity by means of neutral glass filters inserted in the glare source beam.

The variation and determination of the background test spot and glare source intensities followed the same lines as already given in the paper quoted. The multiple decision method was employed for all the l b i determinations.

The subject employed monocular vision for all the measurements covering the unused eye with an eye shade. The head position was kept fixed by making the subject bite on a sealing wax bit. In some cases the subject had to sit at an angle in the cabin and it was found convenient to remove the wooden apron previously employed. Before measurements were commenced the subject sat in complete darkness for at least 45 min and the different stimulations in a complete run were arranged in order to pass from the feeblest to the more intense stimulations.

The complete measurements were made for two subjects W S S employing his left eye (emmetropic) and B H C employing his right eye (slight astigmatic error). The natural eye pupil was used i.e. no artificial pupil or mydriatic was employed. Fixation in any desired direction was secured by fixing the eyes on a weak point source of light consisting of a flash lamp bulb in a short tube which could be pinned at any point on the background screen  $S$ . The intensity of the fixation light was adjusted by the subject so that the light was just visible.

#### RESULTS OF THE MEASUREMENTS

##### *a—Absence of Glare Visual Sensitivity over the Field of the Dark Adapted Eye*

The general character of the visual sensitivity in the fully dark adapted eye for the two subjects (W S S and B H C) was first studied by determining the l b i value in a vertical plane through the fovea centralis and in a plane inclined  $10^\circ$  above the horizontal on the temporal side. An

inclined plane rather than the horizontal was chosen to avoid the blind spot. The data obtained are shown in fig. 3. Here and throughout the paper  $T$  is the  $l b_1$  value in  $c/sq ft$ . It will be noted that for both subjects and in both planes the fovea corresponds to a point of maximum  $l b_1$  or minimum sensitivity but the sharpness of the maximum in the curves is markedly greater for W S S than for B H C. On the temporal side of the curve in the  $10^\circ$  plane B H C exhibits an additional and very sharp maximum at  $\phi$

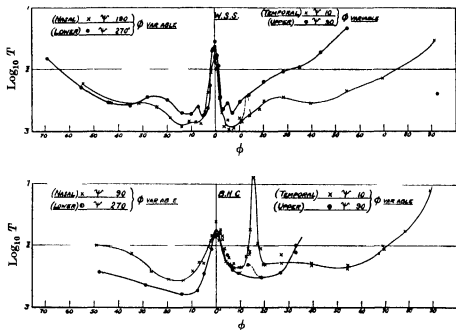


FIG 3

equals about  $16^\circ$ . This is believed to be due to a blood vessel radiating from the blind spot. A corresponding maximum for W S S is not in evidence unless the apparently anomalous point at  $\phi = 13.5^\circ$  is accepted as indicating a smaller maximum of the same type. Other differences between the two subjects' curves are also observable.

#### *b—Effect of Size of Glare Source*

To test the effect of glare source size a comparison was made of angular diameters of the glare source as seen by the subject of  $1.92^\circ$  and  $0.34^\circ$  respectively producing the same illumination  $E_v$  at the subject's eye. The angular positions of test spot and glare source were kept constant during



the comparison. Four measurements were made of the l b i for each size glare source, measurements for the two sizes being made alternately. The results to be given are the means of the four values of  $\log_{10} T$  obtained in this way. Four positions of the test spot were chosen at increasing angular distances  $\phi_T$  from the fixation point and one in each of the nasal, temporal, upper and lower azimuthal planes. For W S S the glare source had a fixed position  $26^\circ$  from the fixation point in the temporal horizontal azimuth ( $\psi_G = 0$ ). For B H C the azimuthal plane was varied. The glare source intensities were chosen to give l b i values within the upper limit of intensity available in the apparatus. Table I summarizes the results obtained.

TABLE I

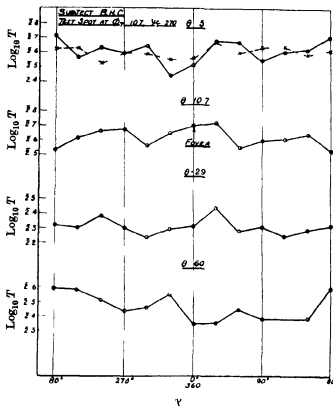
Subject	Test spot position		Glare source position		Glare source intensity $\log_{10} E_T$	Mean $\log_{10} T$		Difference
	$\phi_T$	$\psi_T$	$\phi_G$	$\psi_G$		Large glare source	Small glare source	
W S S	5°	0°	26°	0°	1.30	1.44	1.48	-0.04
	10°	270°	26°	0°	1.30	1.89	1.92	-0.03
	24°	90°	26°	0°	1.42	0.15	0.15	0
	47°	180°	26°	0°	1.42	1.99	0.10	-0.11
B H C	5°	0°	22°	180°	1.62	0.04	0.04	0
	11°	270°	26°	0°	0.47	1.64	1.65	-0.01
	24°	90°	28°	24°	1.81	1.74	1.72	+0.02
	50°	180°	24°	180°	2.67	1.02	1.94	-0.02

Referring to the final column giving the difference between the log of the l b i for large and small glare sources it is apparent that the difference is small (average value -0.024) and we may conclude that, within the range of glare source size studied, the effect on the l b i at extrafoveal points is determined by the eye illumination alone, a similar result to that already obtained by Bordon and others for the case of foveal vision.

*c—Variation of Orientation of the Glare Source with respect to Test Spot at a Constant Glare Angle*

For these tests the glare source was moved round the fixed test spot at a constant angular separation  $\theta$  from the latter, the experiment being carried out for several values of  $\theta$ . The orientation of the glare source with respect to the test spot is expressed by the angle  $\chi$  already defined. In each run measurements of the l b i were made for twelve values of  $\chi$  spaced at  $30^\circ$  intervals. The observations at the twelve  $\chi$  values were taken in random order. For all positions of the glare source (in a given run) the illumination at the eye measured normal to the incident light ( $E_N$ ) was kept constant.

By suitable choice of  $\theta$  it could be arranged that the glare source in its circular path passed through the fovea or the blind spot as it was desired to see if anomalies occurred at these points. In fig. 4 the results of a series of runs with the test spot at the point  $\phi_T = 10.7^\circ$   $\psi_T = 270^\circ$  and with  $\theta = 5^\circ$   $10.7^\circ$   $29^\circ$  and  $60^\circ$  respectively are reproduced (continuous curves). For each value of  $\theta$   $\log_{10} T$  is plotted against the orientation angle  $\gamma$



I 1 4

(N.B.  $\gamma = 0^\circ$  corresponds to the position of the glare source nearest to the fovea  $\gamma = 180^\circ$  corresponds to the most remote position of the glare source) No marked systematic variation of the l.b.i. with the orientation of the glare source with respect to the test spot is apparent in the curves of fig. 4. From reasons of symmetry such variations would be expected to lead to curves symmetrical with respect to an ordinate through  $\gamma = 0^\circ$  if the fovea is regarded as a centre of symmetry in the retina, or with respect to an ordinate through  $\gamma = \text{approximately } 304^\circ$  if the blind spot is taken as the

centre. Axial symmetry about the line joining the fovea to the blind spot would not lead to any easily predictable form of the curve. Assuming no systematic variation with  $\chi$  in the curve of fig. 4 the actual variation from a horizontal straight line may be attributed to experimental errors in determining  $T$ , drift in the value of  $T$  during the run, and irregular variations of the effect of the glare source on the sensitivity at the test spot as the angle  $\chi$  is changed. That the second of these causes plays some part is clear from fig. 5 showing the data of the top curve ( $\theta = 5^\circ$ ) in fig. 4 plotted not against  $\chi$  but in the order in which the measurements were taken. The upward drift in the value of  $\log_{10} T$  during the run is well marked. Drawing the best straight line through the data of fig. 5, a correction to the original observations can be made by adding to each observed value of  $\log_{10} T$  the mean  $\log_{10} T$  for the run, less the value on the straight line of fig. 5 corresponding to the particular observation in question. The dotted

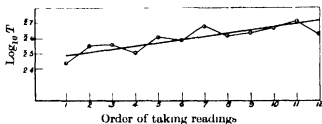


FIG. 5

line in fig. 4 for the  $\theta = 5^\circ$  run represents the data corrected for drift in this manner. The corrected data show less irregularity than the original observations although the difference is not great. In the great majority of the runs for B. H. C. the l.b.i. value drifted upwards during the run. Drift occurred to a less extent for W. S. S. In Tables II and III are given details of all the runs made. Where the number of values of  $\chi$  used is less than 12 (see column 5) this is because the glare source is cut off from the subject's view by the nose or other parts of the face. The difference between the maximum and minimum values of  $\log_{10} T$  recorded in each is shown in column 6, and column 7 gives the average deviation of the  $\log_{10} T$  values from the mean  $\log_{10} T$  in the run.

For B. H. C. (Table II) no systematic change of  $\log_{10} T$  with  $\chi$  was observed for thirteen out of fifteen complete or nearly complete runs (number of  $\chi$  values not less than 9) under different conditions. For four out of the six test spot positions no systematic change occurred. The biggest deviations from constancy of  $\log_{10} T$  occurred with the test spot at  $\phi_T = 50^\circ$ ,  $\psi_T = 180^\circ$ ,

TABLE II—SUBJECT B H C RIGHT EYE

Run	Test spot		$\theta$	Glare source $\log_{10} E_A$	Number of values of $\chi$	Variation of $\log_{10} T$ (uncorrected for drift)		Remarks
	$\phi_T$	$\phi_I$				Maximum spread	Mean devia- tion from the mean	
1	5°	0°	5°	3.38	12	0.17	0.04	No systematic variation
2	5°	0°	10°	3.38	12	0.12	0.02	" "
3	5°	0°	20°	2.33	11	0.19	0.04	" "
4	5°	0°	53°	2.88	9	0.21	0.07	" "
5	10°	0°	5°	3.38	12	0.25	0.04	" "
6	10.7°	270°	5°	3.38	12	0.27	0.06	" "
7	10.7°	270°	10.7°	2.33	12	0.18	0.05	" "
8	10.7°	270°	20°	2.33	12	0.18	0.04	" "
9	10.7°	270°	60°	2.92	11	0.24	0.07	" "
10	23.6°	90°	5°	3.38	12	0.25	0.06	Slight increase of $\log_{10} T$ as glare source moves away from fovea
11	23.6°	90°	23.6°	2.33	12	0.21	0.05	No systematic variation
12	23.6°	90°	10°	2.92	10	0.27	0.08	" "
13	50°	180°	5°	5.52	12	0.66	0.13	Pronounced systematic change in $\log_{10} T$ with maximum in neigh- bourhood of $\chi = 180$
14	50°	180°	5°	5.73	12	0.99	0.22	
15	50°	180°	5°	5.52	12	0.96	0.20	Maximum at about $\chi = 180$ {Data incomplete owing to cut off of glare source by nose, etc
16	50°	180°	10°	3.47	12	0.64	0.11	
17	50°	180°	30°	3.38	6	0.12	0.04	No systematic variation
18	50°	180°	60°	*	7	0.25	0.08	
19	50°	0°	5°	5.73	12	0.30	0.09	" "
20	50°	0°	30°	2.33	12	0.26	0.05	" "

TABLE III—SUBJECT W S S LEFT EYE

Test spot		Glare source $\log_{10} E_A$	Number of values of $\chi$	Variation of $\log_{10} T$ (uncorrected for drift)		Remarks
$\phi_T$	$\psi_T$			Maximum spread	Mean deviation from the mean	
5°	180°	3.36	12	0.19	0.06	No systematic variation
5°	0°	3.36	12	0.23	0.05	" "
5°	0°	3.36	12	0.20	0.05	" "
5°	0°	3.36	12	0.33	0.07	Some systematic variation symmetrical about the ordinate $\chi = 90^\circ$
5°	0°	*	6	0.24	0.07	Data incomplete owing to cut off of glare source
10°	270°	3.36	12	0.13	0.03	No systematic variation
10°	270°	3.36	12	0.20	0.08	" "
10°	270°	2.29	12	0.28	0.07	" "
10°	270°	*	7	0.14	0.04	Data incomplete owing to cut off of glare source
24°	90°	3.36	12	0.30	0.09	Some systematic variation approximately symmetrical about the ordinate at $\chi = 0$
24°	90°	3.36	12	0.25	0.08	
24°	90°	2.29	11	0.45	0.13	
24°	90°	*	6	0.28	0.07	Data incomplete owing to cut off of glare source
48°	180°	3.36	12	0.15	0.03	No systematic variation
48°	180°	3.36	12	0.22	0.04	" "
48°	180°	3.36	9	0.22	0.05	" "
48°	180°	*	6	0.22	0.05	Data incomplete owing to cut-off

\* Not measured

and the data for this case are reproduced in fig 6. The occurrence of a maximum in the neighbourhood of  $\chi = 180^\circ$  for  $\theta = 5^\circ$  and  $10^\circ$  appears to be well established. No corresponding effect is observed if the test spot is  $50^\circ$  to the temporal instead of to the nasal side of the fovea (runs 19 and 20).

For W S S nine out of thirteen complete or nearly complete runs show no systematic variation. With the test spot at  $\phi_T = 24^\circ$ ,  $\psi_T = 90^\circ$  systematic

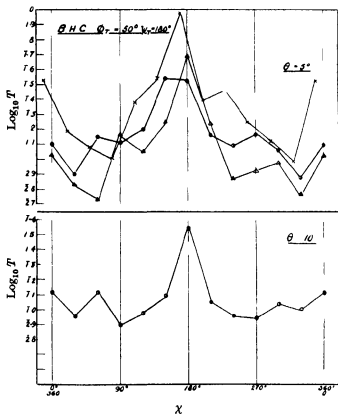


FIG 6

variation occurs in the three complete runs corresponding to different  $\theta$  values and the character of the variation is somewhat similar in the three cases (see fig 7). It may be noted that at the test spot position corresponding to that for which the marked maximum of  $\log_{10} T$  occurred for B H C the curves for W S S show no systematic variation.

Fig 8 shows the results for five runs in which the glare source passed through the fovea in its circular path. The second curve of fig 4 is another such run. Data for three runs in which the glare source traversed the blind

spot are given in fig 9. It appears from these results that neither the fovea nor blind spot corresponds to singular points of the retina at which a glare source will produce an abnormally greater or less effect on the l b 1 than at any other point equally distant from the test spot.

To sum up the results of these tests, it may be said that in most cases the effect of a glare source on the l b 1 at an extrafoveal point shows no systematic

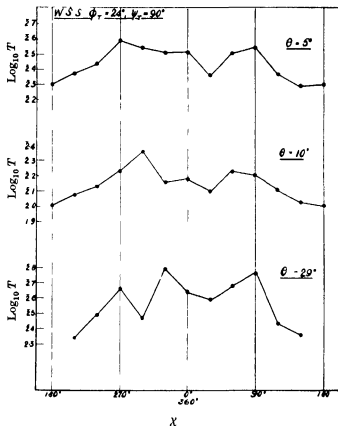


FIG 7

change as the orientation of the glare source with respect to the extrafoveal point is varied. Small irregular changes do occur, however, and in addition at certain extrafoveal points which may be at different positions in the visual field for different individuals the orientation of the glare source has an effect on the l b 1. When the glare source falls on the fovea or on the blind spot the effect on the l b 1 is no different from that for any other point at an equal angular distance from the test spot.

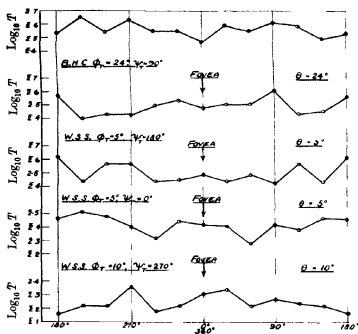


FIG. 8

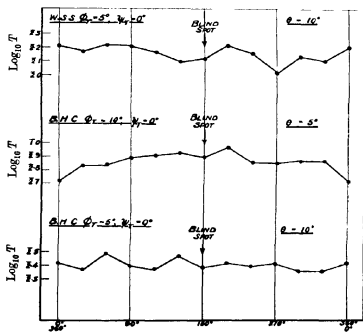


FIG. 9



*d.—Variation of the Intensity of the Glare Source keeping the  
Glare Source and Test Spot in Fixed Positions*

It follows from formula (2) that, with  $B = 0$  and  $\theta$  fixed,  $\beta$  equals a constant multiplied by  $E_N$ , where the constant has the value  $k/\theta^n$ . To test the proportionality between  $\beta$  and  $E_N$  predicted by the formula the following comparison was made for four representative positions of the test spot  $T$  was measured in the absence of glare for a series of uniform field brightnesses  $B$  and the results were plotted as a curve of  $\log_{10} T$  against  $\log_{10} B$ .  $T$  was then measured for various intensities  $E_N$  of a glare source in a fixed

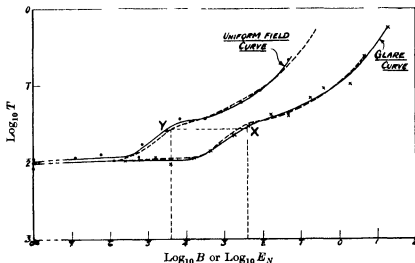


FIG. 10 B H C  $\phi_T = 5^\circ$ ,  $\phi_G = 21.5^\circ$ ,  $\psi_T = 0^\circ$ ,  $\psi_G = 180^\circ$

position with respect to the test spot and the results were plotted as a curve of  $\log_{10} T$  against  $\log_{10} E_N$ . The two curves so obtained in one comparison of this kind (comparison 1) are depicted in fig. 10 (the continuous curves). It is clear that if we take a particular value of  $\log_{10} E_N$ , say  $\log_{10} E_N = 3.6$ , this will correspond on the glare curve to a point  $X$  and to a particular value of  $\log_{10} T$ . Drawing a horizontal line through the point  $X$  to cut the uniform field curve in the point  $Y$ , the abscissa of  $Y$  will clearly equal  $\log_{10} \beta$ , where  $\beta$  is the uniform field brightness giving the same value of  $T$  as the glare source of intensity  $E_N$ . If  $\beta$  is proportional to  $E_N$ , then  $\log_{10} \beta$  must equal  $\log_{10} E_N + \text{const}$  or  $\log_{10} \beta - \log_{10} E_N = \text{const}$ . This means that the length of  $XY$  must be the same whatever the initial value of  $\log_{10} E_N$  may have been, and it follows that the glare curve and the uniform field curve must be similar in shape, with a relative displacement parallel to the

axis of  $\log_{10} E_N$  or  $\log_{10} B$ . The broken line curves shown in fig 10 were obtained by shifting bodily the glare and uniform field curves parallel to the axis of abscissae to bring them respectively into approximate coincidence with the uniform field and glare curves. The agreement between the original and the displaced curves is satisfactory and it follows that for this comparison,  $\beta$  is proportional to  $E_N$  and the prediction of the formula is verified.

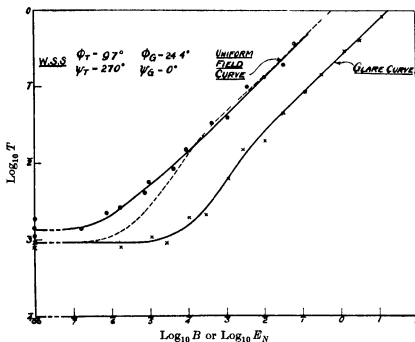


FIG 11

Details of all the comparisons made are given in Table IV. The two curves involved in each comparison were determined on the same day, one in the morning, one in the afternoon. For B H C all four comparisons give satisfactory agreement between the two curves following a suitable displacement of one of them parallel to the axis of  $\log_{10} E_N$  or  $\log_{10} B$ . For W S S two of the comparisons show a discrepancy between the original and displaced curves at low values of  $\log E_N$  or  $\log_{10} B$  which appears to be associated with a change in the condition of the eye between the glare and uniform field runs. The discrepancy is greatest for the comparison illustrated in fig 11.

The observational points on the two curves corresponding respectively to  $\log_{10} E_N = \infty$  and  $\log_{10} B = \infty$  clearly refer to the same stimulation of

TABLE IV

Com- parison	Test spot position		Glare source position		Relative positions of test spot and glare source		Agreement between original and displaced curves	Range of $\log_{10} E_V$	$\log_{10} \beta -$ $\log_{10} E_N$	$\log_{10} k$
	$\phi_T$	$\psi_T$	$\phi_G$	$\psi_G$	$\theta$	$\chi$				
B H C	1	5°	0°	21.5°	180°	26.5°	0°	6.4-1.3	2.12	0.97
	2	10.7°	270°	26°	0°	28.0°	290.6°	6.5-0.8	2.41	1.31
	3	23.6°	90°	28.1°	23.8°	27.8°	67.4°	6.5-0.4	2.26	1.15
	4	49.5°	180°	7.5°	180°	42°	0°	6.4-2.7	2.20	1.44
W S S	5	5°	0°	20°	180°	25°	0°	6.4-1.3	2.20	1.00
	6	9.7°	270°	24.4°	0°	26.2°	290.8°	6.2-1.2	2.52	1.36
7	24°	90°	27.6°	23.6°	27.8°	65.6°	Fairly satisfactory Discrepancy between glare and uniform field curves for $\log_{10} E_V$ less than 5.8 due to shift of 1 b 1 for zero stimulation	6.2-1.6	2.80	1.69
8	49.5°	180°	21.8°	180°	27.7°	0°	Similar discrepancy for $\log E_V$ less than 6.8	6.2-1.5	2.48	1.36
Column (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)

the eye, in fact, to zero stimulation, but there is a significant difference between the values of  $\log_{10} T$  obtained in the two runs. The difference in question makes it impossible to bring the two curves into approximate coincidence by any horizontal displacement although this impossibility cannot be regarded as evidence against the proportionality of  $\beta$  and  $E_v$ . "Drift" or "day to day" variations of the l b i of the kind in question here are familiar and have already been discussed by Stiles (1929) and Stiles and Crawford (1934). They constitute a difficulty in determining equivalent uniform field brightnesses which can be overcome only by repeated determinations of the l b i values extending over a period. Time could not be spared to undertake further work on this point and the conclusion from these tests must be restricted to the following.

The equivalent uniform field brightness  $\beta$  giving the same l b i at a given extrafoveal point as a glare source of fixed position but variable intensity  $E_N$  is proportional to  $E_v$  provided the condition of the eye as evidenced by the value of the l b i when the eye is adapted to zero stimulation remains unchanged.

According to formula (2) the amount by which the glare curve must be displaced to bring it into coincidence with the uniform field curve is equal to  $\log_{10}(k/\theta^n)$ . Column (10) of Table IV shows the values of the displacement required in the eight comparisons. In the two comparisons in which the two curves could not be made to coincide approximately over their whole length owing to the difference in the l b i at zero stimulation the displacement given is that required to fit the curves at the higher values of  $\log_{10} E$  (see fig. 11). The significance of the displacement in these cases is doubtful. Column (11) of Table IV gives the value of  $k$  deduced on the assumption that the observed displacement equals  $\log_{10}(k/\theta^n)$  and that the exponent  $n$  has the value 2.

*e—Variation of the Glare Angle  $\theta$*

To test formula (2) with respect to variation of the glare angle  $\theta$  it was necessary to determine two curves giving the variation of the l b i with uniform field brightness in the absence of glare and the variation with glare angle when a glare source of constant intensity  $E_v$  is moved in a fixed plane containing the test spot direction in such a way as to vary the angle  $\theta$  keeping  $\chi$  constant. The equivalent uniform field brightness  $\beta$  for each value of the glare angle  $\theta$  is determined from these two curves in a precisely similar manner to that already described for determining the equivalent uniform field brightness corresponding to a glare source of fixed position and variable intensity. The results of the experiments are then represented graphically by plotting  $(\log_{10} \beta - \log_{10} E_N)$  ( $E_N$  is constant in each glare

angle run) against  $\log_{10} \theta$ . An example of a graph obtained in this way is shown in fig. 12.

According to formula (2), if  $B = 0$  and  $E_N$  is constant we shall have

$$\log_{10} \beta = \log_{10} E_N + \log_{10} k - n \log_{10} \theta$$

Thus the graph of  $(\log_{10} \beta - \log_{10} E_N)$  against  $\log_{10} \theta$  should be a straight line of slope  $(-n)$  cutting the ordinate  $\log_{10} \theta = 0$  at a value of  $(\log_{10} \beta - \log_{10} E_N)$  equal to  $\log_{10} k$ . In the example of fig. 12 the plotted points lie reasonably

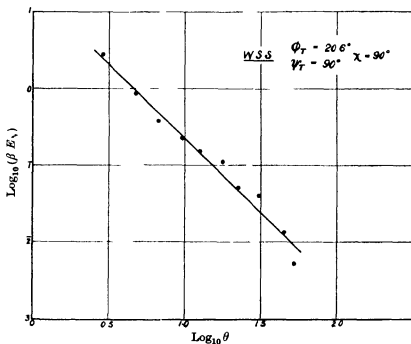


FIG. 12

close to the continuous straight line shown which has a slope of  $(-1.93)$ , the corresponding value of  $\log_{10} k$  being 1.28.

Table V gives the details of the runs made for different positions of the test spot for the two subjects. Except for the measurements with the test spot at  $5^\circ$  in the parafovea, the data are based on a uniform field curve determined in a single run, and on one or two glare angle runs. For the  $5^\circ$  position more extensive measurements were made and the graphs of  $\log_{10}(\beta/E_N)$  against  $\log_{10} \theta$  based on the mean curves of three or four uniform field and glare angle runs for each subject are shown in fig. 13.

An examination of column (7) of Table V shows that the values of  $n$  deduced in each case from the slope of the best straight line through the

points of the appropriate graph similar to fig. 12 range from 1.77 to 2.35 with mean values for the four test spot positions equal to 1.97 and 2.04 for the two subjects. No claim for great precision can be made for measurements of this kind but we are justified in concluding that our results show the approximate validity of formula (2) as representing the effect of variation of the angle of glare with a value of the constant  $n$  which is certainly nearer

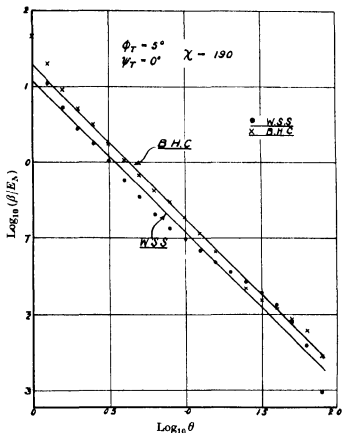


FIG. 13

to 2 than to 1.5 or 2.5 and for which the value 2 may be assumed as representative. The corresponding values of  $\text{log}_{10}k$  (column (8) of Table V) vary from 0.78 to 1.50 with means of 1.18 and 1.19 for the two subjects. If instead of drawing the best straight line to fit the variation of  $(\text{log}_{10}B - \text{log}_{10}E)$  with  $\text{log}_{10}\theta$  we draw the best straight line of slope  $(-2)$  we get the  $\text{log}_{10}k$  values shown in column (9) of Table V. The mean values for the two subjects are 1.21 and 1.17 respectively.

TABLE V

Test spot position $r = \text{about } 5.5 \text{ ft}$			Glare source position relative to test spot $r = 3.8 \text{ ft}$		Glare source intensity $\log E_v$	$n$	$\log_{10} k$	$\log_{10} k$ for $n = 2$
B	H	C	$\phi_T$	$\psi_T$	$\chi$	Range of $\theta$		
	5°		0°		190°	1 80°	4.76	2.02
	10 7°		270°		270°	1 92°	4.76	1.77
	23 6°		90°		45°	2 90°	4.76	2.04
	50°		180°		350°	7 5 91°	4.76	2.04
							Means	1.97
	5°		0°		190°	12 102°	5.30	—
W	S	S	5°	0°	190°	1 80°	4.76	1.99
	9 7°		270°		270°	1 92°	4.66	2.35
	20 6°		90°		90°	3 92°	4.66	1.93
	48°		180°		10°	4 95°	4.74	1.87
							Means	2.04
	5°		0°		190°	12 105°	5.30	—
Column (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)

As a representative value of  $\log_{10} k$  we may take  $\log_{10} k = 1.20$  or  $k = 16$ . The values of  $\log_{10} k$  ( $n = 2$ ) given in column (9) of Table V and also those given in column (11) of Table IV are plotted against  $\phi_T$  the angular distance of

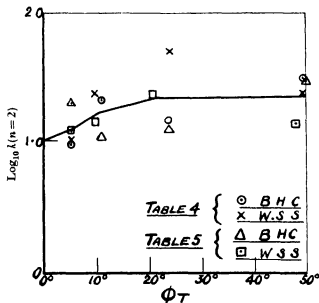


FIG 14

the test spot from the fixation point, in fig 14. The straight line graph shown in fig 16 was obtained by joining up the mean values of  $\log_{10} k$  at the different angles including the value  $\log_{10} k = 1$  for  $\phi_T = 0$  (foveal vision) which is taken as a representative value from previous investigations. Unfortunately the scatter of the  $\log_{10} k$  values is too great to reveal with certainty any variation of  $\log_{10} k$  with the angle  $\phi_T$  although we may perhaps conclude that  $\log_{10} k$  is somewhat greater for extrafoveal than for foveal vision.

Some additional measurements of the variation of the l b i with glare angle were made for the  $5^\circ$  test spot position at very large glare angles  $\theta$  up to  $100^\circ$  and with a more powerful glare source than was used for the main runs to obtain a greater glare effect at large angles. This was done in order to see what happens when the glare source moves up to the limit of the field of vision. The derived values of  $\log_{10}(\beta/F_v)$  are plotted against  $\log_{10} \theta$  in fig 15.

The quantity of light from the glare source which is admitted to the eye by the pupil must clearly diminish as the glare source moves away from the fixation direction i.e. as  $\phi_G$  increases. In the absence of the corneal refraction the apparent area of the pupil from the glare source direction would equal the apparent area seen from the fixation direction multiplied by  $\cos \phi_G$ . Owing to the corneal refraction the factor  $\cos \phi_G$  must be modified and the pupil will still have an area different from zero when  $\phi_G$  equals  $90^\circ$ . The angle  $\phi$  at which the apparent pupil area vanishes depends to some extent on the absolute size of the pupil and for a large pupil size (7 or 8 mm) is of the order of  $105^\circ$ . If  $\phi_G$  exceeds the limiting angle any effect of the glare source on vision must be due to light diffusing through the sclera or possibly to secondary glare sources formed by the illumination of the nose, eyelids or orbit. In the absence of effects of this kind which we should expect to be

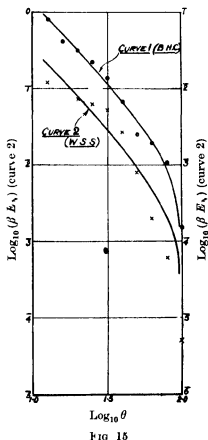


FIG 15



small the equivalent uniform field brightness must become zero whatever the intensity of the glare source when  $\phi_G$  exceeds a value of the order of  $105^\circ$ . According to formula (2), it is clear that, putting  $B = 0$ ,  $\beta$  cannot vanish unless  $E_N$  equals zero. The modification to the formula which suggests itself is to multiply  $E_N$  by the ratio  $R(\phi_G)$  of the apparent pupil area as seen from the glare source position to the apparent pupil area as seen from the fixation point. The formula would then become

$$\beta = B + k \frac{E_N R(\phi_G)}{\theta^n}. \quad (4)$$

The ratio  $R(\phi_G)$  has been calculated from the dimensions and refractive properties of the eye\* and the computed values have been used to determine the relation between  $\log(\beta/E_N)$  and  $\log_{10}\theta$  for a test object at  $\phi_T = 5^\circ$ ,  $\psi_T = 0^\circ$  and for a glare source in the azimuthal plane  $\chi = 190^\circ$ , putting  $B = 0$  and  $n = 2$ . Choosing  $\log_{10}k$  suitably, the continuous curves in fig. 15 represent the relation between  $\log_{10}(\beta/E_N)$  and  $\log_{10}\theta$  arrived at in this way. It is seen that for one subject (B. H. C.) the experimental points can be fitted closely with the computed curve. For the other subject the agreement is less good. Some further investigation of the effect of glare at very large angles is desirable.

The authors have pleasure in acknowledging the assistance rendered by Messrs F. W. Cusack, B.Sc. and H. R. Sayer in making the measurements given in this paper.

The work was carried out under the auspices of the Illumination Research Committee of the Department of Scientific and Industrial Research.

#### SUMMARY AND CONCLUSION

The results of this investigation go to show that, within the range of the variable factors studied, the effect of a glare source at any point in the visual field on the l.b.i. at any extrafoveal point is, in general, the same as that produced in the absence of the glare source by a uniform field of brightness  $kE_N/\theta^n$  where  $n$  and  $k$  have values equal approximately to 2 and 16 respectively,  $\theta$  is the angle between glare source and test point and  $E_N$  is the illumination of the eye from the glare source. No abnormal effect on the l.b.i. is observed when the glare source falls on the blind spot or on the fovea centralis. It appears however that for certain positions of the test spot, different in different eyes, the effect of a glare source at a given

\* A note on these calculations will be published elsewhere.

glare angle  $\theta$  may depend on the orientation of the glare source with respect to the test spot. When the glare source approaches the limit of the field of view it is probable that the glare effect is determined not by the eye illumination  $E_N$  but by the product of  $E_N$  and where  $R$  is the ratio of the apparent area of the pupil viewed from the glare source position to its apparent area viewed normally. At small angles,  $R$  differs little from unity.

The special difficulties of extrafoveal measurements of the l b i and the "drift" or 'day to day' variations of the l b i make an exact test of the equivalence of a glare source and of a uniform field brightness impossible, but we may conclude that for practical purposes the expression  $kE_N/\theta^n$  can be used with confidence in estimating the order of magnitude of the effect of a glare source on extrafoveal vision.

The fact that if we take any two points of the retina the effect of a light source imaged at one point on the sensitivity at the other point as measured by the l b i, can be represented by a relatively simple formula for the equivalent uniform field brightness containing only two constants is remarkable and suggests a corresponding simplicity in the mechanism of the glare effect. The view that the equivalent uniform field brightness corresponds to an actual illumination of the retina by scattering of the rays from the glare source in some part of their path in the eye, is capable of explaining qualitatively the main features of the results obtained. However, difficulties are met with when a quantitative comparison between theory and experiment is attempted. A detailed discussion of the mechanism of the glare effect is reserved for a later communication.

#### APPENDIX

##### *Best values of the constants $k$ and $n$ in the formula for equivalent uniform field brightness for foveal vision*

The values originally obtained by Luckiesh and Holladay (1925) were  $k = 12.7$ ,  $n = 2$ . A further series of measurements by Holladay (1927) gave  $k = 9.2$ ,  $n = 2$ . Stiles (1929) obtained  $k = 4.2$ ,  $n = 1.5$  but worked down to an angle  $\theta = 1^\circ$  at which the glare source actually encroached on the rather large test object ( $2^\circ$  diam.).

In fig. 16 are plotted the values of  $\log_{10} m$  against  $\log_{10} \theta$  taken from Table XI of the paper cited, where according to the formula, the coefficient  $m$  should equal  $k/\theta^n$ . The straight line previously drawn through the points corresponds to  $k = 4.2$ ,  $n = 1.5$  (dotted line in fig. 16), but actually the data are better fitted by the curve shown which is asymptotic for large values of  $\theta$  to the straight line corresponding to  $k = 7$ ,  $n = 1.8$ . The deviation of the

plotted points from this line at the smaller glare angles may be attributed

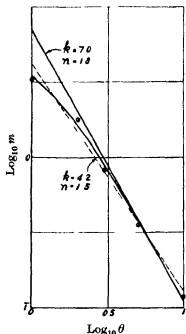


FIG 16

to the fact that at these angles there is a marked variation of visual sensitivity across the test spot which tends to overweight the parts of the test spot remote from the glare source and thereby to increase the effective glare angle. An approximate estimate of the possible deviation due to this cause shows it to be of the right order of magnitude to explain the observed results. Thus, as revised values of  $k$  and  $n$  appropriate to glare angles not too small compared with the test spot dimensions, we may take 7.0 and 1.8 respectively. A similar correction to Luckiesh and Holladay's results is too small to be worth applying.

A large number of approximate determinations of  $k$  and  $n$  have since been made by Crawford and Stiles (1935) using a special instrument for the measurement of equivalent uniform field brightnesses. The means of all these results give  $k = 11.5$  and  $n = 2.09$ . The different values are collected together in Table VI.

TABLE VI

	$k$	$n$
Luckiesh and Holladay (1925)	12.7	2.0
Holladay (1927)	9.2	2.0
Stiles (revised values)	7.0	1.8
Crawford and Stiles (1935)	11.5	2.1
Means	10.1	1.98

Thus as approximate representative values for  $k$  and  $n$  for foveal vision we may take  $k = 10$ ,  $n = 2$ .

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# CORRIGENDA

D KEILIN and E F HARTREE "On some properties of catalase haematin," 'Proc Roy Soc ' B 121, 1936

The numbers in the last four columns of Table I (p 177) and in the second line of the summary (p 190) should be divided by 10

In column 6 Table I, for 'haematin' *read* 'haematin Fe'



## On the Responses of the African Migratory Locust to Different Types of Background

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(Received 16 January 1937)

### INTRODUCTION

It is known that the hoppers (or immature forms) of certain species of locusts, in the solitary phase, bear a marked colour resemblance to the immediate environment in which they are living. They are, for example, green when living among fresh green vegetation, light brown on sand, speckled greyish white on white limestone rocks, and so on. Faure (1932) conducted a series of experiments with *Locustana pardalina* (Walk.) and *Locusta migratoria* subspecies *migratorioides* R. and F. in South Africa. His object was to discover whether there was only a coincidence between the prevailing coloration of the hoppers of these two species and that of their environment, or whether a genuine colour adaptation is involved. He isolated a number of young hoppers, of the same stock, placing each insect in a separate wooden box, provided with a sliding glass lid and reared it to the adult instar. The wood of each box was painted on the inside with ordinary commercial oil paints and, when dry, the latter were compared with the coloured plates of Ridgway (1912) and named according to the tints which they resembled most closely. The experiments of Faure were done out of doors, and he recorded the general coloration assumed by each locust during and at the end of the experiments, noting them as "good", "fair", "slight" or "none", according to the degree of resemblance their coloration bore to that of the insides of the boxes. He obtained a majority of "good" or "fair" resemblances on white, black, grey, yellow and brown backgrounds in the boxes, and one striking resemblance on a black and white striped background. No resemblance occurred when confined in boxes painted green, pink, blue, or with black and orange stripes. Faure also carried out some other experiments on a smaller scale, such as using glass lids of the colours resembling those of the walls of the boxes, but these, as he states, were, on the whole, inconclusive. He brought forward evidence,

however of great importance as regards the production of green hoppers. These he found did not result in response to a green background and were only produced in the presence of a moist atmosphere and an abundance of succulent food. He further showed that the green colour producing substance was spectroscopically very different from chlorophyll and prepared extracts showed none of the characteristic fluorescence.

The present paper describes experiments which have been made with a view to determining the relation between the general coloration of the hoppers and the wave lengths of the effective colours forming different backgrounds.

#### MATERIAL AND METHODS

The locusts used in this work were *Locusta migratoria* subspecies *migratoria* R. and F. which is kept breeding from generation to generation in the Entomological Field Station at Cambridge. The original stock was obtained as eggs from near Khartoum in 1933. For the purpose of the present investigation only the progeny of locusts in the gregarious phase was used. They were kept in a small glasshouse where the temperature was controlled by means of a thermostat; this prevented cooling down below a minimum level but at times during bright sunshine the temperature rose considerably. The mean temperature during the experiments was approximately 32°C. The relative humidity in the glasshouse was kept as near to 60% as possible. In each colour test fifteen locusts were used and four tests involving sixty locusts were going on at the same time. Each locust was placed as early as possible after hatching from the egg in a separate Petri dish (10 cm. in diameter and about 2 cm. in height) where it remained until the end of the fifth instar. In order to allow for increase in the size of the locusts during growth the lid of each dish was kept slightly raised by means of wire when the insects reached the third instar. Each batch of fifteen Petri dishes forming a single experiment was placed in a tray measuring 53 x 32 cm. and 9 cm. high. The four trays employed simultaneously were lined with papers of different colours. The locusts were fed once daily with grass which has proved to be a satisfactory food under the conditions stated. The humidity within the dishes varied according to whether the grass was obtained during wet or dry conditions. If the conditions within the Petri dishes became unduly humid there was an increased tendency for the locusts sooner or later during development to become green as Faure had already observed. If on the other hand conditions became too dry the mortality among the locusts increased. The

green forms, as will be referred to later, were significant from the present standpoint, owing to the fact that parts of the abdomen and legs showed colour changes in general conformity with those forms which exhibited the full colour response

The coloured papers used for lining the trays were the standard Ostwald papers manufactured in Leipzig. The following fourteen colours were employed (of the full-coloured Na series)

Ostwald No	2 = Light cadmium of Ridgway
" "	4 = Salmon orange "
" "	6 = Scarlet "
" "	7 = Spectrum red "
" "	11 = Pansy violet "
" "	12 = Spectrum violet "
" "	13 = Phenyl blue "
" "	14 = Spectrum blue "
" "	16 = Oxide blue "
" "	18 = Italian blue "
" "	21 = Skobeloff green "
" "	22 = Emerald green "
" "	23 = Yellow green "
" "	24 = Greenish yellow "

In addition to these colours, white, black, grey and also striped backgrounds were used. The black was a very rough paper, apt to absorb light as completely as possible. Black and white backgrounds were produced by painting a black pattern on the undersides of the Petri dishes, which were then placed in a tray which was lined with white paper. After the colour tests had been carried out, seven of the coloured papers employed, which had given the most significant results, were examined for their spectral properties by Messrs Adam Hilger and Co., Ltd., of London. The spectrum reflected from the coloured surface was compared photometrically with that reflected from a magnesium oxide surface which is considered to represent an absolute white. The curves shown in figs. 1 and 2 express the percentage of reflected light of different wave-lengths. In all these experiments as much direct light as possible was admitted and the dark framework of the glass roof over the experimental table was concealed by means of a "ceiling" of white paper. Only on a few occasions, when there was a risk of the Petri dishes becoming too hot and dry, bright sunshine was screened off by an outside blind. In another set of experiments, instead of normal daylight, only coloured light was allowed to fall on the Petri dishes. Two colour screens, made of cellophane, stretched over frames, were used: one of these was yellow and the other violet. We are indebted to Dr C. B. Allsopp, of the Physical Chemistry Laboratory, Cambridge, for determining the



percentage of light of different wave lengths transmitted by the cellophane (*vide* fig 3) The cellophane was replaced twice by new sheets in order to preclude the risk of its fading during the experiments

Only the visible part of the spectrum is represented in figs 1-3 because the ultra violet of normal daylight was not considered to be a factor in these experiments since it would have to pass through the glass of the roof of the house and the lids of the Petri dishes before reaching the eyes of the locust Even less of any possible ultra violet light would reach the eyes of the locusts by reflection, since it has to pass twice through the glass bottoms of the Petri dishes before it can exert any possible influence

The colours assumed by the hoppers as the result of the different experiments are described in terms of the Ridgway (1912) colour standards A final statement was made when the insects attained the end of the fifth instar Owing to the fact that no locust is quite uniformly coloured, personal judgement has to come in when describing their colour type The face, for example, is always brighter than the rest of the body, the under parts of the abdomen are paler than the upper and there is a variable pattern always to be taken into account It needs to be pointed out, however that the colour effects were described from observations made simultaneously and by M Hertz throughout Locusts from different coloured trays were compared with one another as well as with the Ridgway standards and in this way it is hoped that inaccurate comparisons have been avoided

During the experiments those hoppers which died during the first 2 or 3 days were replaced by others of the same age those dying later were not replaced

#### EXPERIMENTS WITH WHITE ILLUMINATION (DAYLIGHT) ON DIFFERENT BACKGROUNDS

*I Hoppers on Black, White, Grey and Striped or Spotted Background—* These experiments were carried out during February 1936 when the daylight was poor No hoppers died after the first instar

(a) *Black background* two hoppers were sooty black four dark grey, mottled with black two brownish (ranging from snuff brown to saccardos umber and sepia, R XXIX\*) seven became green, more or less mottled with dark shades

(b) *White background* five hoppers very pale (Tilleul buff) in the fourth instar, became light brownish in the fifth instar (ranging from wood brown

\* These symbols refer, in all cases, to the plates in Ridgway (1912)

to avellaneous and vinaceous buff, R XL) seven hoppers, colonial buff and ivory yellow (R XL, XXX) in fourth instar, became green, sometimes more or less whitish green in fifth instar

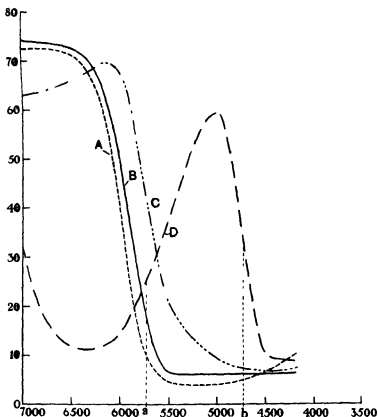


FIG. 1.—Percentages of rays of different wave length in the reflected light from backgrounds of A, spectrum red, B, scarlet, C, salmon orange, and D, emerald green (In this fig., and in figs 2 and 3, *a* and *b* mark respectively the positions of purest yellow and blue for the human eye. The ordinates denote percentages and the abscissae show wave lengths in Å.)

(c) *Neutral grey background* five hoppers were more or less pure grey, of the shade of the background three showed more colour (different shades of drab, R XLVI) seven became more or less green

(d) *White and black background* the background was either striped or spotted (the stripes and spots ranging between 3 and 6 mm in diameter) no conspicuous pattern was noted on the hoppers four hoppers were pure grey five more or less brownish grey of the same shades as those in (c). six became green

II *Hoppers on Salmon Orange Pansy Violet Black or Grey Background*—These experiments were carried out during March and April 1936 when the daylight was better. No hoppers died after the second instar.

(a) *Orange background* many hoppers became green during early development six became orange brown during third and fourth instars the bright parts of the body such as the face were ochraceous orange while other parts were ochraceous tawny to buckthorn brown (R XV). Of these six hoppers one retained this coloration until the end of the fifth instar while the others became green all the green hoppers had orange brown stripes on the abdomen and orange brown legs.

(b) *Violet background* no hopper developed any orange or yellow five became pure grey (darker shades of mouse grey or neutral grey R LI and LII) until the end of the fifth instar with some brown only on the wings and considerable pattern. Remaining hoppers became green with the legs and stripes on the abdomen pure grey without any orange.

(c) *Black background* all the hoppers became sooty black with the exception of three one of these latter was behind in development and was brownish the other two were green greatly spotted with black.

(d) *Grey background* hoppers became very similar to those in I (c) the grey colour was not so pure or dark a grey as those on violet background.

III *Hoppers on Skobeloff Green Emerald Green Yellow Green and Greenish Yellow*—This experiment was carried out during April and May. There was a slight increase in mortality.

(a) *Skobeloff green background* hoppers began to lose colour during the third instar in the fourth instar the difference between those on this blue green background and those on other green colours began to be very conspicuous. In the fifth instar eight hoppers were rather pure grey (light olive grey to olive grey R LI) four hoppers were more brownish (drab) (R XLV) with a tendency to become green three hoppers were blue green with pure grey stripes on the abdomen and grey legs.

(b) *Emerald green background* one hopper became the most brilliant orange (ochraceous orange R XV) noted in any of these experiments ten hoppers showed different shades of orange brown (clay colour wood brown chamois honey yellow R XXIX XXX) two were very light (deep colonial buff R XXX) four were yellow green with orange brown stripes on the abdomen and a similar colour on the legs.

(c) *Yellow green background* all the hoppers were light or pale no orange coloured examples developed six ranged from chamois to honey yellow.

and clay colour, R XXX, XXIX) four were green with brownish stripes on the abdomen and a similar colour on the legs.

(d) *Greenish yellow background* six hoppers ranged from ochraceous tawny and ochraceous buff (R XV) to naples yellow and mustard yellow (R XVI), these colours varying in darkness seven hoppers were green with orange brown legs

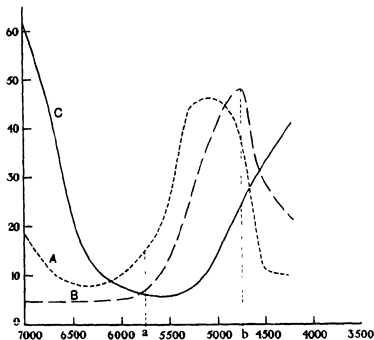


FIG. 2—Percentages of rays of different wave length in the reflected light from backgrounds of A, Skobeloff green, B, Italian blue, and C, spectrum violet

IV *Hoppers on Spectrum Red, Phenyl Blue, Spectrum Blue and Oxide Blue*—The experiments with these colours were carried out during May when general conditions were drier fewer hoppers became green but the mortality was increased

(a) *Spectrum red* the hoppers developed a rather dark greyish colour without any orange or brown until the fifth instar. In this instar they began to develop shades of brown, five becoming bone brown and three were somewhat paler, snuff brown to olive brown (R XL, XXIX) two became dark grey and one blue-green with grey stripes on the abdomen and grey legs.

(b) *Phenyl blue* one hopper developed a slight indication of brownish colour while all the others became pure grey—rather pale with darker

pattern—until the fourth instar. At the end of the fifth instar eleven were uniformly more or less dark grey.

(c) *Spectrum blue* without exception the hoppers became pure grey. At the end of the fifth instar eleven became grey of lighter shades than in the case of IV (b) where the blue background is more reddish. Two became green with pure grey stripes on the abdomen and grey legs.

(d) *Oxide blue* the hoppers became very pale (pale olive grey R LI) of these ten had a much darker greyish pattern while one hopper showed a slight trace of brown.

V *Hoppers on Light Cadmium Yellow Scarlet Spectrum Violet and Italian Blue*—During these experiments which were made in June the food was very dry and only one hopper developed green coloration. At the end of the month very hot weather with bright sunlight impinging on the lids of the Petri dishes caused the death of many of the hoppers. Since the majority had reached the fifth instar the experiment was closed.

(a) *Light cadmium yellow* no very bright or full colours developed and seven hoppers became yellowish old gold honey yellow or chamois (R XVI XXX).

(b) *Scarlet* the colour response developed rather slowly. In the fourth instar and at the beginning of the fifth instar nine hoppers assumed the duller shades of orange brown buckthorn brown ochraceous tawny or clay colour (R XV XXIX).

(c) *Spectrum violet* the hoppers became very pure grey differing as to shades of darkness in different individuals. One hopper became green with pure grey stripes on the abdomen and pure grey legs.

(d) *Italian blue* the hoppers became a very light pure grey three of them were almost whitish some darker but all had a distinct dark pattern on the thorax.

On the whole in Exp V the hoppers developed more pattern than in any of the other experiments. Two individuals one on scarlet and the other on light cadmium yellow showed so much dark pattern that in general appearance their coloration was very near that assumed by hoppers in the gregarious phase.

VI *Hoppers in Wire and Zinc Cages*—Since the experiments in which white and black striped or spotted backgrounds (No 1) were used had given no result which might confirm Faure's suggestion that the pattern of the background might be reproduced to some degree by the hoppers further tests were carried out. These were done with the object of increasing the differences in the brightness of the patterns employed. Twenty cylindrical

cages, each 20 cm high and 10 cm in diameter, were made from three different types of perforated zinc and from fine wire netting. The perforated zinc had holes 2, 4, and 8 mm in diameter respectively, the intervening metal between the holes was approximately 1, 2 and 3 mm in width, respectively. The wire netting was of 0.5 mm wire, the meshes being 1.5 mm across. Each cage was lined with transparent colourless cellophane in order to preclude the escape of the young hoppers. The twenty cages were placed upon a white background in as full daylight as possible. Owing to the height of the cages, the hoppers were able to move away from the immediate vicinity of the food and thus rest in a somewhat drier atmosphere. This appears to account for the fact that none of the twenty hoppers used in the experiments turned green.

The results of these experiments are given below—the colour assumed by each hopper in the fifth instar being recorded.

(a) *Wire netting cages*

- (1) Dark grey, with very little pattern
- (2) Similar to (1)
- (3) Very uniformly grey
- (4) Similar to (3)
- (5) 'Drab', dark pattern only on thorax

(b) *Perforated zinc cages (2 mm holes)*

- (1) Dark grey, with but little pattern
- (2) 'Drab', dark pattern only on thorax
- (3) Similar to (2)
- (4) Similar to (2) but with pattern on thorax slightly more distinct
- (5) Similar to (4)

(c) *Perforated zinc cages (4 mm holes)*

- (1) Dark grey, with very little pattern
- (2) Light grey, with very little pattern
- (3) 'Drab', with dark pattern on thorax
- (4) Similar to (3), but with dark pattern slightly more distinct
- (5) Similar to (4)

(d) *Perforated zinc cages (8 mm holes)*

- (1) Very uniformly grey
- (2) Grey, very little pattern
- (3) 'Drab', very little pattern
- (4) Similar to (3)
- (5) Similar to (3), but with more pattern on thorax

None of the hoppers in the foregoing experiments (VI) showed any marked differences from those reared on a uniformly coloured background. The different background patterns afforded by the cylinders therefore cannot be stated to have exercised any noticeable influence.

VII *Effects of Background Colours on Newly Hatched Hoppers*—In the experiments already described it was constantly observed that the first instar hoppers varied considerably in coloration among themselves—before being used for experimental purposes. The following experiment was undertaken with the object of determining whether this variability is an hereditary factor or whether it is due to environmental or other influences which became operative soon after the hoppers emerged from the eggs. Four lots of hoppers of ten individuals each which had just issued from the eggs and had not as yet developed any coloration were taken. Each lot was placed in a Petri dish without food and the four dishes were placed respectively on backgrounds of salmon orange, pansy violet, white and black. After 48 hours pronounced colour differences developed. Hoppers on the white background all became much lighter coloured than those on the black; those on pansy violet background all became pure grey and only those on orange background showed any orange coloration. The hoppers were then isolated, each being placed in a separate Petri dish and fed in the usual manner. Those which were placed originally on orange and on violet backgrounds were now transferred to grey (near to neutral grey of Ridgway) while those which were originally on black or on white backgrounds were transferred to black. The differences between the four original lots remained well defined up to the end of the second instar. In the third instar these differences completely disappeared and the hoppers showed an evident assimilation to the new backgrounds.

The experiment proves that the optical effect of the background is very strongly marked in newly hatched hoppers during the first 2 days—when the first colour response becomes evident. If the background be changed during later instars it requires much longer than 2 days before a noticeable difference in coloration develops.

VIII *Crowded Hoppers on a Blue Background*—It has been stated in a previous experiment that certain blue backgrounds result in the production of very pale grey hoppers without any development of orange. The blue background colour obviously inhibits the development of orange yellow. In order to ascertain the possible effect of such a background on the coloration of the hoppers in the crowded condition, twenty hoppers in the first instar were placed in a glass cage on a background of Italian blue.

Ostwald paper. No noticeable influence was observed. The crowd developed the normal coloration assumed by hoppers in the gregarious phase. In this connexion it may be noted that in none of the hoppers reared under gregarious conditions in Cambridge is quite the same coloration developed as described by Faure in South Africa. Faure (p. 354) identifies the orange colour in the hoppers studied by him as being orange rufous of Ridgway (II). Under the conditions in Cambridge the orange coloration is always less brilliant and paler and comes nearest to ochraceous orange of the Ridgway series (XV).

#### EXPERIMENTS WITH VARIED ILLUMINATION ON VARIED BACKGROUNDS

*I Hoppers kept on White or Black Backgrounds under Yellow or Violet Illumination*—Two trays similar to those previously used were lined with white and the other with black paper. They were covered with a frame across which was stretched transparent yellow cellophane. In this way predominantly yellow light (see fig. 3B) was transmitted to the Petri dishes in the trays and reflected by the background. In the same way a frame of violet cellophane (fig. 3A) was placed over two other trays lined with black and with white paper. The number of hoppers and of Petri dishes used were the same as those described in the previous experiments with backgrounds of various coloured papers.

It needs to be mentioned that for a few minutes daily the hoppers were exposed to daylight while the food was changed in the dishes.

On black background under both yellow and violet screens the hoppers developed were much the same coloration as those kept on black background under rather poor daylight illumination (Exp. I(a)). Many of the hoppers became green but two under the violet screen and a similar number under the yellow screen became sooty black during the third or fourth instar. The remainder developed orange brown in dull and dark shades of a varied character.

On white background not a single hopper developed either a pure grey or black colour under yellow or violet screens. They all became more or less dull shades of orange brown during the third and fourth instars.

*II Hoppers kept in the dark*—Two of the usual trays lined with black paper were placed one over the other so as to form a lightproof dark chamber. Fifteen hoppers each in a separate dish were reared under these conditions. They were exposed to the light for a few minutes daily during renewal of the food. The hoppers soon became pale coloured and were a very light colourless grey at the beginning of the third instar. Subsequently



they began to develop, very slowly, some coloration, becoming drab grey and light drab (R XLIV) to avellaneous and wood brown (R XL). In the fifth instar all the hoppers turned more or less green, evidently owing to the effect of the high humidity, induced by increased amount of food, under the confined conditions to which they were subjected

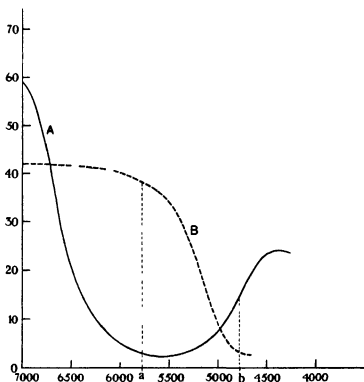


FIG 3—Percentages of rays of different wave length in the transmitted light through screens of A, violet cellophane and B, yellow cellophane

#### DISCUSSION OF THE RESULTS

In the foregoing experiments, with the unique exception of hoppers reared on black, no complete adaptation to the colour of the background was observed in any instance. Nevertheless, the effects of the background colours are very clear. The hoppers reared upon various backgrounds may be described as being colourless or coloured. By colourless is implied the development of more or less black, so that the hoppers assumed different shades of a pure grey. Coloured hoppers were more or less orange yellow, blended with a variable amount of black pigmentation, with the result that

they assumed many different shades of darker or paler purer or duller coloration

The Ridgway colour standards have a chromatic scale of thirty six saturated or full colours from which more than 600 paler or darker dull or broken colours are derived by the addition of greater or lesser amounts of neutral grey black or white. The colours developed by the hoppers on backgrounds of scarlet salmon orange light cadmium yellow green greenish yellow and emerald green are without exception to be found in the Ridgway series under three main series only (i.e. 15 17 and 19). This indicates that the colour component in the coloration of the hoppers is invariably a mixture of 80 % orange and 20 % yellow or 65 % orange and 35 % yellow or 47 % orange and 53 % yellow. On the other hand the non colour component varies very greatly and is responsible for the hoppers appearing in different tints or shades in the sense used by Ridgway. Other colours were only observed in the hoppers in very few cases. On spectrum red background for instance a very dark brown was noted (bone brown). This has a colour component of 91 % orange and 9 % yellow (series 13) together with a large non colour component. Among hoppers reared on Skobeloff green a very pale grey (light olive grey) coloration was assumed. This grey comes under the Ridgway series 23 which implies a small colour component of 100 % yellow together with a large non colour component. The lightest coloured hoppers or those which developed the smallest amount of black were reared on a background of the Ostwald colour paper yellow green (No. 23).

The colours of the hoppers reared (a) in the dark (b) on a white background under yellow or violet illumination (c) on a white background in full illumination together with those which did not become black or pure grey on black or grey backgrounds are found also in the Ridgway colour series 15 17 19 and 21. The palest individuals of the coloured group were reared on a white background or in the dark the former developed relatively more orange yellow and the latter relatively more black.

The hoppers which as a rule never developed any colour but assumed different shades of pure grey were reared upon the following backgrounds—pansy violet spectrum violet phenyl blue spectrum blue oxide blue and Italian blue. The differences in paleness or darkness as the case may be were not very regular or conspicuous but taken as a whole the darkest hoppers of this group were reared on pansy violet background and the palest hoppers on oxide blue and Italian blue.

It may be stated therefore that the optical conditions of the background colour and illumination strongly influences the amount and proportion in

which black and orange yellow is developed. The actual colours involved, however, are none other than those normally present in hoppers reared under ordinary conditions in the gregarious phase. In order to ascertain which of the light rays reflected from different backgrounds, are responsible for inducing the colour responses in the hoppers, the spectra obtained from the different papers used were compared. The graphs for Italian blue and spectrum violet (fig. 2 B and C) represent spectra which exercised no colour effect. The spectra of the other blue and violet papers are not figured, since they would only differ in the amount of reflected violet and red. By the shapes of the curves B and C in fig. 2 it is concluded that those rays which stimulate the production of the orange yellow coloration of the hoppers must lie between the limits of yellow i.e. approximately 5500 and 6000 Å. If there are rays inhibiting the production of orange yellow such rays must lie outside these limits. On Italian blue only very pale hoppers are produced: the greater part of its rays lies between 5000 and 4500 Å and therefore this part of the spectrum may be regarded as a colour inhibitor. With Skobeloff green (fig. 2 A), which produced pale but not quite colourless hoppers, only a small part of its spectrum lies between 5500 and 6000 Å: the blue part of its spectrum is also weak. The graphs for scarlet and salmon orange and emerald green represent the most effective colours (fig. 1 B (C, D)). They each include a large percentage of rays between 5000 and 6000 Å. Emerald green (fig. 1 D) has a very high percentage of rays of about 5500 Å, and it is evident that when inhibition occurs of orange yellow it is due to rays considerably shorter than these. The fact that spectrum red produces only dark coloured hoppers suggests that rays longer than 6000 Å are not effective either in colour production or in colour inhibition. The effect of a red background is the same as that of a very dark surface.

The region of the spectrum which is common to the most diversely shaped curves of effective colour producing spectra, viz. emerald green and scarlet, is the actual part which represents pure yellow (5770 Å) for the human eye.

It has just been mentioned that hoppers reared on Skobeloff green and spectrum red backgrounds show very slight coloration. Those on the former background, however, show more yellow and those on the latter more orange than hoppers reared on papers of the intermediate colours. These facts appear to indicate that rays between 5500 and 5770 Å favour the development of yellow and those between 5770 and 6000 Å favour that of orange.

The normal mixed daylight, when reflected from a white background, of course contains a large proportion of yellow and, therefore, colour producing rays, but the hoppers do not develop a bright orange coloration

in this case. On the contrary they appear in these experiments very pale and dull coloured. The conclusion is therefore drawn that mixed daylight contains a range of colour inhibiting rays which can only be (in accordance with the results of other experiments) those shorter than 5000 Å.

The amount of black coloration of the hoppers seems to depend only upon the difference in the intensity of the light incident from above and reflected from the background. On a black background nearly all of the locusts became black when the illumination was very bright daylight with frequent sunshine, only very few became black when the illumination was rather poor daylight or if the light was transmitted through the yellow or violet screens.

In the same way the high proportion of black admixed with the coloration of the hoppers reared on spectrum red shows that the longest rays of the visible spectrum are ineffective or dark. The pale or bright appearance of those living on blue proves that the rays near the opposite end of the visible spectrum are very bright for the locust. For the same reason yellow green appears also to be very bright for the insect.

The fact that the hoppers become dull shades of orange brown when light that contains no wave lengths of 5500–6000 Å (violet screen p 291) is reflected from a white background requires explanation because the optical effect exercised from the background alone is in this case very similar to the effect of white light reflected from a violet background (compare figs 3 A and 2 C). The only difference lies in the qualities of the incident light which is violet in the one case and white in the other. With white light impinging on a violet background there is a remarkable contrast between the incident and reflected light and pure grey hoppers result. With violet light impinging on a white background there is no such contrast and orange brown hoppers are produced. The conclusion is therefore drawn that contrast between the incident and reflected light is necessary to effect control of the coloration of the hoppers and so to bring about adaptations to the adjacent background.

With regard to the production of green hoppers it needs to be stressed that such forms do not result in response to green or other coloured background. They are produced as the result of high humidity and are independent of background influence. The production of green forms can be absolutely precluded by lowering the humidity to the requisite degree. These dry conditions however entail such high mortality that the value of the experiments becomes vitiated. The balance between the right humidity to keep the hoppers alive and healthy and at the same time to preclude the production of green forms has been found difficult to attain.

consistently under the conditions of these experiments. This appears to be due to the threshold of humidity, leading to the production of green forms, differing in individual hoppers. While the green hoppers do not show a background response in so far as their general coloration is concerned, certain parts of the body, e.g. the legs and the longitudinal stripes on the abdomen, are frequently coloured differently. It will be noted on reference to the records of many of the experiments (pp. 286-288) that the legs and abdomen, in the green forms, assume the same general coloration as is betrayed by non-green hoppers exhibiting the full background response. The effect of a given background, therefore, is betrayed to a limited degree in the coloration of green hoppers, and the latter may, consequently, be regarded as supplementary indicators in the experiments concerned.

Hoppers with the most striking patterns did not result from experiments using conspicuous patterns as backgrounds (Exps. I (d) and VI) but from accidental conditions involving external heat and drought (p. 288). No confirmation is afforded, therefore, of Faure's suggestion that the pattern, sometimes developed by locusts living under solitary conditions, is controlled by the optical effects of the background.

In connexion with the foregoing experiments with *Locusta*, reference needs to be made to results obtained by various investigators with another Orthopteroous insect, viz. *Carausius (Dixippus) morosus* (Fam. Phasmidae). In Germany the colour changes shown by this insect have attracted the attention of several recent workers, notably Giersberg (1928), Atzler (1930) and Priebatsch (1933). *Carausius* is able to produce more or less pigmentation according to the nature of the background, as is the case in *Locusta*. It is, furthermore, able to respond to sudden changes in background coloration by movements of the pigment granules in the hypodermal cells. The pigment granules become aggregated or diffused according to whether the insect becomes paler or darker in its general colour. The darkening of the animal depends upon differences in the intensity and quality of the light incident from above and reflected from below. When the under-parts of the eyes are covered with black paint the same darkening effect is produced as with a black background. On the other hand, when black paint is applied to the upper halves of the eyes, or over the whole of these organs, no such result is obtained. The incident light is most effective when composed of short waves and is less effective when long waves are used. *Carausius* may become either green or brownish in different degrees of darkness, but it never becomes pure grey on blue or violet backgrounds, or brilliantly coloured on orange or yellow surfaces. Its capacity for colour adaptation in these directions, therefore, is less perfect than is the case with *Locusta*.

The primary sensation of the background coloration is received through the eyes and acting on the visual centre of the brain apparently induces the secretion of a hormone into the blood. It would appear therefore that it is this hormone which activates the movements of the pigment granules. Destruction of the optic ganglia or of the tritocerebral region of the brain causes all colour response to cease. In the case of *Locusta* it is also very probable that the colour effects operate through the eyes. This conclusion is supported by the fact that a black background in bright illumination gives an absolutely different effect as compared with darkness. It is also probable that hormones are produced through visual stimulation which control the development of the different pigments.

#### SUMMARY

1—Hoppers (nymphs) of the African migratory locust were reared separately upon different backgrounds from the first instar up to the end of the fifth instar.

2—With the unique exception of hoppers reared upon a black background no complete colour adaptation was observed but the effects of different backgrounds were clearly defined.

3—The coloration of hoppers reared under solitary conditions is fundamentally the same as that of hoppers in the gregarious phase. None other than black and orange yellow is produced.

4—The background only influences the amount and proportion of the orange yellow and black produced. If a large proportion of yellow rays (5500–6000 Å) is reflected from the background the production of orange yellow in the hoppers is much stimulated. If there are no rays of this kind but a larger proportion of those shorter than 5000 Å the hoppers become colourless, pale grey. If the amount of these shorter rays is not so great the hoppers become a darker grey.

5—If only very little light of rays shorter than 6000 Å is reflected but a larger amount of longer rays the hoppers become dark brown.

6—The foregoing statements refer to incident rays composed of mixed white daylight which result in a definite contrast being produced between incident and reflected light.

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## Preparation of Pure Cytochrome *c* from Heart Muscle and some of its Properties

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### INTRODUCTION

Theorell described recently (1935-1936) a large scale method of preparation of cytochrome *c* from ox heart.

According to this method minced muscle is defatted with acetone and benzol, dried, pounded, washed three times with water and extracted with decinormal sulphuric acid. The extract is neutralized, the precipitate centrifuged off and the fluid considerably reduced in bulk by evaporation. The solution is then fractionated with ammonium sulphate giving finally cytochrome containing 0.17% of iron which is about 50% pure. Further purification was carried out by adsorption on barium sulphate and elution with decinormal HCl or by adsorption on cellophane, which is sectioned with a freezing microtome and eluted with dilute ammonia.

The final steps of purification according to Theorell, give samples of cytochrome containing 0.34% of iron. The yield of this material, which Theorell rightly considers to be pure cytochrome, is approximately 1 g. per 100 kg. of heart muscle.

In this paper we describe a new and much simpler method for the preparation of pure cytochrome *c* with an iron content of 0.34%. This new method has the advantage of eliminating the laborious preliminary stages of defatting and drying the muscle pulp, the reduction of the first extract to a small bulk and the final stages of adsorption on cellophane, sectioning the latter, and elution.

Our method consists only in direct extraction of freshly minced heart muscle with trichloroacetic acid followed by a fractional precipitation with ammonium sulphate and trichloroacetic acid. The yield of pure cytochrome by this method is considerably higher than by Theorell's method and corresponds in our estimation to about 70% of the total cytochrome *c* in the heart muscle.

#### SMALL-SCALE PREPARATION

One ox heart is carefully freed from fat and ligaments and minced very finely with a Latapie mincer. After pressing out the blood as far as possible in a hand press, the pulp 1100 g is mixed with 1100 c.c. 0.15 N\* (2½ %) trichloroacetic acid and allowed to stand at room temperature for 2 hr. with occasional stirring. The pH of this mixture is approximately 4. The fluid is pressed out, neutralized to about pH 7 with caustic soda, and centrifuged for 10 min. The clear fluid 1330 c.c. so obtained shows strong absorption bands of reduced cytochrome together with weak oxyhaemoglobin bands. It is treated with ammonium sulphate (50 g. per 100 c.c.) filtered and the filtrate, 1700 c.c., now free from haemoglobin, is treated again with ammonium sulphate (5 g. per 100 c.c.) and left overnight in an ice chest. The pH of the mixture is about 4.9. Next day the liquid is filtered and, while still cold, treated with one fortieth of its volume of 20 % trichloroacetic acid bringing the pH of the mixture to 3.7. Within 10 min. the spectrum of reduced cytochrome disappears and the cytochrome is completely precipitated in the oxidized form. The suspension is centrifuged for 10 min. the bright red deposit shaken with 500 c.c. saturated ammonium sulphate solution and centrifuged again. The red solid is transferred to a cellophane sac by means of about 20 c.c. distilled water and the mixture dialysed for 2 days at 4° C. against 1 % sodium chloride solution. The content of the sac is shaken with a few drops of chloroform and filtered to yield about 30 c.c. of a clear dark red solution containing 0.182 g. pure cytochrome the iron content of which is 0.34 %.

#### LARGER SCALE PREPARATIONS

For preparation on a larger scale, the Latapie mincer is replaced by a "No. 500" geared mincer,† giving a very fine mince upon which the success of the extraction depends. About 6 kg. of minced muscle of ox heart which corresponds to about six hearts can be easily extracted under ordinary laboratory conditions by following the method described above. This amount of mince gives nearly 1 g. of pure cytochrome. For most of our work, however, cytochrome was prepared from horse heart muscle which, besides being easier to handle, invariably gave a much higher yield. Thus, 6 kg. of mince obtained from 3 to 4 hearts gave 1.58 g. of

\* Checked by titration using phenolphthalein.

† This mincer is supplied by G. Rushbrooke, Ltd., Charterhouse Street, London, E.C. 1.



pure cytochrome *c* with an iron content of 0.34% i.e.  $1.6 \times 10^{-4}$  g atom iron of cytochrome per kg of muscle. The only difference which can be noticed during this preparation is that after neutralizing and centrifuging the first extract it remains slightly opalescent. This difference however does not interfere with the further steps of purification.

#### ESTIMATION OF PURITY OF CYTOCHROME *c* IN SOLUTION

The solutions thus obtained are of a dark brownish red colour and show a strong absorption spectrum of oxidized cytochrome. On reduction with  $\text{Na}_2\text{S}_2\text{O}_4$  the colour of the solution turns bright pink and the absorption spectrum of oxidized cytochrome is replaced by the two characteristic strong bands of reduced cytochrome *c*. No change in this absorption spectrum is apparent on addition of either pyridine or haematin thus showing that the cytochrome solution is free from unbound haematin and also from nitrogenous substances capable of giving haemochromogen with free haematin.

The purity of cytochrome is estimated from the ratio of iron to the dry weight obtained after removal of sodium chloride from the solution by dialysis. As cellophane strongly adsorbs cytochrome during dialysis in absence of salts it is necessary either to dialyse against dilute ammonia (0.880 ammonia diluted 200 times with distilled water) which is ultimately removed in a desiccator over sulphuric acid or to dialyse it in parchment diffusion shells against distilled water.

Some loss due to adsorption by parchment takes place during this dialysis but this loss is comparatively small.

The iron in the salt free solution of purified cytochrome is estimated by the  $\alpha\alpha$ -dipyridyl method (Hill and Keilin 1933) which consists in destructive oxidation by means of alkaline  $\text{H}_2\text{O}_2$  of the tetrapyrrolic nucleus of cytochrome. The iron thus liberated is treated with  $\alpha\alpha$ -dipyridyl in presence of a reducer. The resulting red colour is compared with a series of standard tubes containing from  $10^{-5}$  to  $10^{-4}$  g atom iron per l and differing from each other by intervals of 5%.

Estimated in this way the iron in salt free solutions of purified cytochrome was found to represent 0.34% of the total solids which agrees with the highest value obtained by Theorell. The equivalent weight of cytochrome *c* is thus 16,500.

A preliminary investigation has been made by Mr G. S. Adair of the osmotic pressure of cytochrome *c* solutions in M/15 phosphate buffer pH 6.8 using small volumes of dilute solutions. Only a rough approximation

to osmotic equilibrium was obtained as a certain amount of cytochrome was absorbed by the membranes. The provisional results for the molecular weight agree within 10 % with the equivalent weight determined by iron analysis.

Attempts were made to fractionate cytochrome of this purity (1) by adsorption on gelatinous calcium phosphate and elution with alkaline phosphate ( $\text{Na}_2\text{HPO}_4$  or  $\text{Na}_3\text{PO}_4$ ) (2) by fractional precipitation with trichloroacetic acid in presence of ammonium sulphate and (3) by precipitation with lead acetate. In no case however were fractions obtained in which the iron content exceeded 0.34 %.

The concentration of cytochrome *c* in various fractions of the preparation can be easily estimated from the height of the absorption bands of the reduced pigment. Spectrophotometric examinations of pure cytochrome have confirmed the results previously obtained by Dixon, Hill and Keilin (1931) namely that for the absorption at 5497 Å

$$\log_e \frac{I_0}{I} = cd \times 0.62 \times 10^8$$

where *c* is the concentration of cytochrome iron in g. atom per c.c. and *d* is the depth of liquid examined. Using a Hilger Nutting spectrophotometer giving direct readings of  $\log_{10} \frac{I_0}{I}$  and cells 2 cm. long

$$c = \log_{10} \frac{I_0}{I} \times 1.86 \times 10^{-8}$$

Samples of stock solutions to be estimated are accurately diluted 30-50 times with very dilute  $\text{Na}_2\text{HPO}_4$  and reduced with  $\text{Na}_2\text{S}_2\text{O}_4$ .

TABLE I

Preparations	Purity of cytochrome %	Yield of cytochrome in g. 100 kg muscle	g. atom of cytochrome Fe extracted/kg muscle
Theorell (1935) ox heart	100	1	$6 \times 10^{-4}$
Theorell (1936) ox heart	50	4.15	$2.5 \times 10^{-4}$
Described in this paper			
(a) ox heart	100	16.5	$10^{-4}$
(b) horse heart	100	26.4	$1.6 \times 10^{-4}$

The results of these estimations of purified samples agree within 3-4 % with the estimations of total iron by the  $\alpha\alpha$ -dipyridyl method.

PREPARATION OF CYTOCHROME *c* FROM A HOT AQUEOUS EXTRACT  
OF HEART MUSCLE (KOCHSAFT)

1500 g of minced horse heart muscle is mixed with 1500 c.c. of 0.002 N acetic acid and slowly heated to 80° C with stirring. After keeping at this temperature for 10 min. the mixture is pressed out through cloth, cooled, and neutralized with NaOH to pH 7.3. The fluid is filtered through a fluted Chardin paper giving 2 l of a clear fluid containing a large amount of cytochrome *c* and a little haemoglobin. This fluid is treated with 10.13 g ammonium sulphate and 41.6 c.c. 0.02 N ammonia. The mixture is filtered and the slightly cloudy filtrate (2380 c.c.), is treated with 119 c.c. 20 % trichloroacetic acid.

On centrifuging, a pink cake is obtained which is washed with saturated ammonium sulphate solution, suspended in 20–30 c.c. water and dialysed against 1 % sodium chloride solution until completely free from sulphate. 1 c.c. of this solution has dry weight (loss on ignition after drying) of 17.6 mg and contains 2.5 mg of cytochrome.

The preparation obtained in this way is only 14 % pure and yields 18.3 g of cytochrome *c* per 100 kg of muscle. Such a preparation, although it can be used for most of the experiments with cytochrome, is not as suitable as the previous one for the isolation of the pure pigment.

PROPERTIES OF PURE CYTOCHROME *c*

Pure cytochrome *c* obtained from heart muscle has the same properties and the same absorption spectra in the oxidized and reduced states as cytochrome *c* extracted from baker's yeast. We shall examine here only a few properties of cytochrome *c* which have not been previously described, and we shall discuss also some of our previous results which have not been generally accepted.

(1) Strong solutions of pure cytochrome *c* examined spectroscopically have never revealed the presence of the two additional absorption bands in the red region of the spectrum described by Bigwood and his co-workers (1933). It is obvious that these two bands do not belong to cytochrome *c*. They belong probably to an oxidation product of either cytochrome *c* or of some other intracellular haematin compound.

In fact we could find these bands only in a few old preparations of cytochrome obtained from yeast and kept at room temperature exposed to air. In one case a solution turned green, and the only bands then present were those at the red end of the spectrum.

(2) Oxidized and reduced cytochrome *c* are trivalent and divalent iron compounds respectively. The transition from reduced to oxidized cytochrome is a true oxidation reaction accompanied by a change in valency of the iron.

Contrary to the views expressed by Shibata and his co-workers (1930) cytochrome does not combine loosely with molecular oxygen and never forms oxygenated compounds similar to oxyhaemoglobin. In fact oxidized cytochrome *c* which shows a typical parahaematin absorption spectrum can be kept a long time and even boiled in a vacuum tube in complete absence of oxygen without undergoing reduction.

(3) Reduced cytochrome does not easily oxidize in air and does not combine with CO within the physiological range of pH. It can be shaken with oxygen in complete absence of reducing substances without undergoing oxidation. On prolonged shaking or long standing in air it will gradually oxidize especially if the sample is not pure. Such oxidation is however very slow and has no biological significance. For all practical purposes we can consider cytochrome *c* as a non autooxidizable haematin compound.

(4) Cytochrome becomes autooxidizable however below pH 4 when the parahaematin spectrum changes into that of acid haematin. On the alkaline side it becomes distinctly autooxidizable at pH 12. Reduced cytochrome *c* begins to combine with CO at pH 12.5 and forms a CO compound at pH 13. This compound is very sensitive to light, rapidly dissociating when exposed to light during spectroscopic examination and reforming in the dark. On neutralizing the alkaline solution of cytochrome it reverts completely to its original state and loses the properties of reacting with oxygen and carbon monoxide.

(5) Reduced cytochrome *c* as was previously shown (Keilin 1930) is easily oxidized by potassium ferricyanide, copper salts and hydrogen peroxide. The oxidation by  $H_2O_2$  however was recently denied by Theorell (1935) who found that  $H_2O_2$  only destroys cytochrome. We have found that the destructive action of  $H_2O_2$  is only marked when an excess of  $H_2O_2$  is added to an alkaline solution of cytochrome and this reaction has been used for the liberation and estimation of cytochrome iron (Hill and Keilin 1933). In a slightly acid solution a very small concentration of  $H_2O_2$  oxidizes reduced cytochrome without destroying it.

(6) Biologically cytochrome *c* is oxidized only by indophenol oxidase and reduced by some dehydrogenase systems. Catechol oxidase however has no effect on reduced cytochrome.

(7) On boiling a solution of oxidized cytochrome *c* it turns yellow brown and the parahaematin absorption spectrum disappears completely. On

cooling, the colour and absorption spectrum return to the original state. The boiling and cooling accompanied by these changes in colour and absorption spectra can be repeated several times without affecting the properties of cytochrome. The reversible change in the absorption spectrum of oxidized cytochrome *c* produced by warming is a general property of all the para-haematin compounds. It is probably due to a temporary dissociation of the nitrogenous compound from the haematin nucleus. The latter, liberated as neutral haematin, does not show a distinct absorption spectrum.

On the other hand, boiling the reduced cytochrome *c* in complete absence of oxygen has no effect on its colour and absorption spectrum. Only in presence of a trace of oxygen does the reduced cytochrome become rapidly oxidized on boiling, and behaves then as a true para-haematin compound.

(8) Cytochrome *c* does not form compounds with substances such as cyanide, sulphide, fluoride, azide, hydroxylamine or peroxides which combine with methaemoglobin, catalase, and peroxidase.

(9) The only substance with which cytochrome *c* reacts and forms a reversible compound is nitric oxide.

#### REACTION OF CYTOCHROME WITH NO

The study of this reaction was carried out in complete absence of oxygen in Thunberg tubes from which oxygen was removed by evacuating the tube, washing it several times with pure nitrogen, and then filling it with pure NO drawn from a mercury gasometer.

Three tubes containing brownish red solutions of oxidized cytochrome at pH 5, 6, and 7 and showing distinct absorption spectra of the para-haematin type are filled with NO in absence of oxygen. The colour of the solutions in all cases changes almost immediately to a carmine red and the para-haematin absorption spectrum is replaced by two very strong bands  $\alpha - 565 m\mu$  and  $\beta - 530 m\mu$  (fig. 1).

The reversibility of the reaction can be demonstrated as follows. Oxidized cytochrome brought to pH 5 with acetate buffer (4 c.c.) is placed in a Thunberg tube with 0.05 c.c. 16 % sodium sulphite. On treating the solution with NO and shaking it for a few seconds, the NO-cytochrome spectrum appears. On standing, this absorption spectrum is replaced within 2 min. by that of oxidized cytochrome, owing to absorption of NO by the sulphite. On shaking the solution again the NO cytochrome compound reappears, and then disappears on standing. This reversible change can be observed four times, after which a mixture of reduced cytochrome and the NO compound is obtained.

For experiments with reduced cytochrome and NO an even more rigid exclusion of oxygen is essential owing to the strong oxidizing action of  $\text{NO}_2$  on cytochrome. The experiment is therefore carried out in a double limb Thunberg tube one limb of which contains reduced cytochrome solution at pH 7 while the other contains alkaline  $\text{Na}_2\text{S}_2\text{O}_4$  solution which removes the last traces of oxygen. On filling the tube with pure NO and

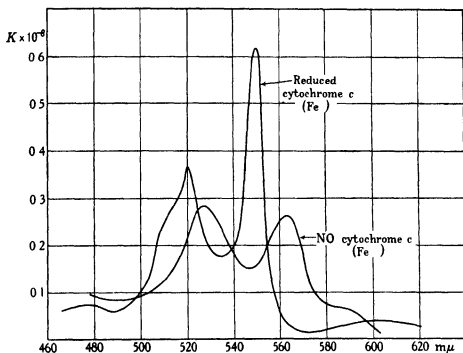


FIG 1.—Absorption spectrum of NO cytochrome compound compared with that of reduced cytochrome. Ordinate: absorption coefficient per gram atom of cytochrome iron. abscissa: wave length in  $m\mu$ .

shaking it the reduced cytochrome remains unchanged. Admission of a little oxygen leads to the immediate and complete formation of the NO cytochrome compound.

It is thus apparent that NO combines only with oxidized cytochrome, giving a well defined compound with two distinct absorption bands at 565 and 530  $m\mu$ . We have noticed, however, that if a little  $\text{Na}_2\text{S}_2\text{O}_4$  is added from the hollow stopper of the Thunberg tube to NO cytochrome compound the absorption bands shift to 572.5 and 532.5  $m\mu$ , become very diffuse and are very slowly replaced by those of reduced cytochrome. This compound

may be either the reduced haematin of cytochrome or the reduced NO cytochrome compound which cannot be obtained however from reduced cytochrome treated with NO

#### DETERMINATION OF SOME AMINO ACIDS IN CYTOCHROME *c*

The comparatively easy method of preparation of pure cytochrome *c* enabled us to prepare a sufficient amount of material for the determination of a few amino acids of this compound

#### ACID HYDROLYSIS OF CYTOCHROME *c*

103 c.c. of cytochrome solution containing 1440 mg. of cytochrome was refluxed for 4 hr. with twice its volume of concentrated hydrochloric acid in an all glass apparatus and then evaporated to dryness. The residue was dissolved in water, evaporated to dryness twice, the dark residue boiled with distilled water (300 c.c.) and the suspension treated with hot baryta solution until alkaline to thymol phthalein. The flocculent brown precipitate was filtered off and the barium in the filtrate and washings precipitated at 100° by its equivalent of sulphuric acid. After filtering off the barium sulphate a practically colourless solution was obtained and in this way the use of charcoal or other decolorizers with the consequent risk of loss of amino acids was avoided.

The separation of the mono amino acids from diamino acids was effected by evaporating the solution to dryness and following the method described by Hanke and Koessler (1920) using 100 c.c. 15% phosphotungstic acid. The solution of diamino acids was finally made up to 1 l. (solution A). Cystine was estimated in solution A by the method of Fohn and Marenzi (1929), arginine by the method of Jean (1934) and histidine by the method of Jorpes (1932). The total nitrogen in A was determined by the Kjeldahl method and the lysine by difference assuming that A contained only the four diamino acids. The results of these determinations compared with the average results obtained by previous authors on horse haemoglobin are given in Table II.

TABLE II

Amino acids	% in cytochrome	% in haemoglobin
Arginine	5.6	3.57
Cystine	1.1	0.74
Histidine	7.8	8.13
Lysine	9.1	8.31
Tryptophane	0.9	2.38

TRYPTIC DIGESTION OF CYTOCHROME *c* AND THE DETERMINATION  
OF TRYPTOPHANE

20 c c solution containing 213 mg cytochrome *c* was incubated in presence of toluol and chloroform for 8 days at 37° C with 5 c c dilute ammonia (prepared by diluting 0.880 ammonia forty times) and 0.2 g fresh pancreatin. The solution was then boiled, filtered, cooled and made up to 50 c c with distilled water. It was reddish brown in colour. The tryptophane was estimated colorimetrically by the method of Komm and Bohringer (1924) and the percentage was found to be 0.94.

SUMMARY

A simple method is described for the preparation of pure cytochrome *c* from heart muscle. The method consists only in extraction of heart muscle with trichloroacetic acid and fractional precipitation of the extract with ammonium sulphate.

By this method 10 kg of heart muscle of ox or horse give respectively 1.65 and 2.64 g of pure cytochrome with an iron content of 0.34 %. The equivalent weight of cytochrome *c* was found to be 16,500. The molecular weight of cytochrome determined by osmotic pressure (Adair) was found to be of the same order of magnitude.

The absorption spectrum and other properties of cytochrome *c* extracted from heart do not differ from those of cytochrome *c* obtained from baker's yeast.

Cytochrome *c* as was previously shown is not easily autoxidizable and does not combine with CO within the physiological range of pH. It reacts with oxygen and CO only at pH above 12. The oxidation of reduced cytochrome is accompanied by a change in the valency of its iron. Reduced and oxidized cytochrome are therefore divalent and trivalent iron compounds.

Cytochrome never forms with oxygen a loose oxygenated compound similar to oxyhaemoglobin.

Biologically cytochrome *c* is specifically and very rapidly oxidized by the tissue oxidase (indophenol or cytochrome oxidase).

The properties of cytochrome *c* are not affected by repeated boiling and cooling.

Cytochrome does not combine with cyanide, sulphide, fluoride, azide and peroxides. Oxidized cytochrome forms a reversible compound with NO and this compound has a very characteristic absorption spectrum composed of two distinct bands at 565 and 530 mμ.



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## A Comparative Study of the Brains in Pleuronectidae

BY H MUIR EVANS

*(Communicated by Sir Henry Dale, F R S —Received 24 June 1936)*PART I THE MEDULLA OBLONGATA, AND ITS VARIATIONS  
ACCORDING TO DIET AND FEEDING HABITS

In previous communications to this Society the relationship of the habits of feeding and diet to the form and pattern of the medulla oblongata has been described in the cyprinoids, clupeids, and gadoids (Evans, 1931, 1932, 1935) This research takes up a similar study of the brain of the Pleuronectidae The expense has been borne by a grant from the Royal Society for which the author tenders his grateful thanks It has seemed to be desirable to extend the observations to the fore- and mid-brain, as in some members of the family these present a very marked development In order to elucidate some of the problems that arise I have also studied the brain of the eel, and some interesting conclusions have resulted.

We find, as a result of examination by the naked eye and of serial sections, that we can divide the following species into four groups as follows

- I The sole, *Solea vulgaris*
- II The plaice, *Pleuronectes platessa*, the lemon sole or lemon dab, *Pleuronectes microcephalus*, the witch, *Pleuronectes cynoglossus*, the dab, *Pleuronectes limanda*
- III The turbot, *Rhombus maximus*, the brill, *Rhombus laevis*, the megrim, *Arnoglossus megastoma*
- IV The halibut, *Hippoglossus vulgaris*

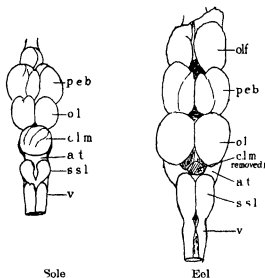


PLATE I

Lettering of Plates I-IV

*peb* primitive end brain, *ol* optic lobes, *clm* cerebellum, *at* acoustic tubercles, *ssl* somatic sensory lobes, *v* vagal lobes, *olf* olfactory bulbs, Halibut, inferior aspect, *li* lobus inferior, *sv* saccus vasculosus, *pg* pituitary gland, *on* optic nerve, *med* medulla

The asymmetry of the head, the upward aspect of the eyes, and the crossing of the optic nerves have been fully discussed by many authorities, but as, for practical purposes, those parts of the brain which concern us are nearly symmetrical, I do not propose to make any further reference to these characteristic features

Outline drawings of the brains of groups I-IV

Group I is typified by the sole, the brain of the eel has been included for purposes of comparison. In the sole the primitive end brain is very large,

the optic lobes are small the somatic sensory lobes are large and globular and have been wrongly described as facial lobes In the eel the primitive end brain and olfactory bulbs are still larger the optic lobes are somewhat larger than in the sole the somatic sensory lobes are more elongated and their anterior extremities are not so globular

*Group II* The plaice has a smaller primitive end brain than the sole the optic lobes are very large and each is convoluted so as to make it bilobed the medulla compared with that of the sole is less prominent and more elongated and the rhomboid fossa is narrow and slit like The brains of the lemon sole the witch and the dab are very similar to that of the plaice but the optic lobes are not bilobed although in the lemon sole a prominent lateral bulge is seen anteriorly

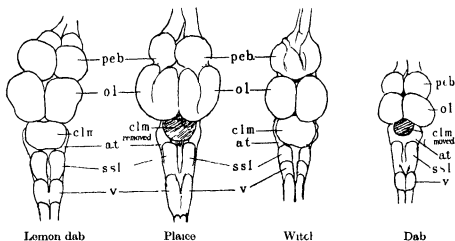


PLATE II

*Group III* The turbot may be taken as a type The olfactory bulbs and corpus striatum are markedly smaller than the corresponding areas in the previous two groups but the optic lobes are large the somatic sensory lobes are more rounded and especially in the turbot are wider and the medulla oblongata is not so elongated It will be noted that in none of these groups does the facial lobe appear on the surface although in group II we shall see that it is quite well developed The picture of the brain in group III is very similar to that which I have described in those Gadidae that have largely a diet of fish

*Group IV* The brain of the halibut presents a similar picture to that of the turbot but the optic lobes are somewhat bilobed

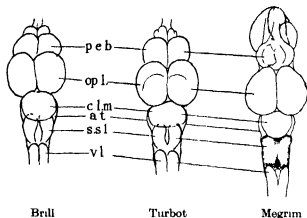
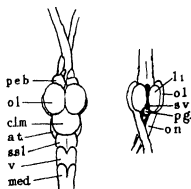


PLATE III



Halibut

PLATE IV

#### TECHNIQUE OF HISTOLOGICAL EXAMINATIONS

As in my previous communications, the brain was fixed in Bouin's fluid and then hardened in successive strengths of alcohol. The sections were stained with Delafield's haematoxylin and counter-stained with picro-indigo-carmin. The formula of the picro-indigo-carmin used was

A Saturated solution of picric acid in 90 % alcohol

B Saturated solution of indigo-carmin in 70 % alcohol

Mix in the proportion A 1 volume, B 1 volume, 70 % alcohol 6 volumes

## THE MEDULLA OBLONGATA OF THE SOLE

In the physiological series of the *Catalogue of the Royal College of Surgeons* (Burne 1902) the medulla is described as 'fairly long with a pair of very prominent lobes (facial?) upon its dorsal surface' The suggestion that these lobes are facial is not borne out by a study of serial sections Com

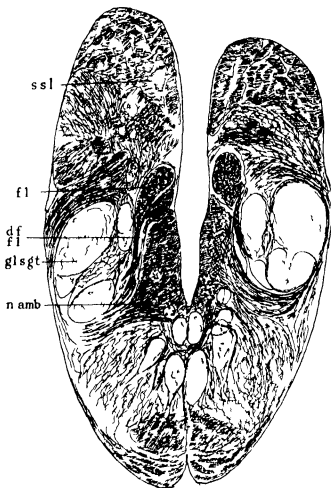


FIG 1—Sole

## Lettering of Figures

*fl* facial lobe, *gls gt* great longitudinal secondary gustatory tract, *n amb* nucleus ambiguus, *mtv* motor tract of vagus *ssl* somatic sensory lobe, *clm* cerebellum, *sm* stratum moleculare, *sg* stratum granulosum, *cal* central acoustic lobe, *VIIIn* facial nerve, *dfV*, descending fibres of Vth nerve, *at* acoustic tubercle, *dfVIIIn* descending fibres of facial nerve, *vent* ventricle, *n r* nuclei rotundi, *li* lobi inferiores

mening at the caudal extremity the vagal lobe is seen to be rather narrow and not prominent. Anterior to the vagals the facial lobes appear as small spherical areas (in section fig 1) resting on the dorsal extremities

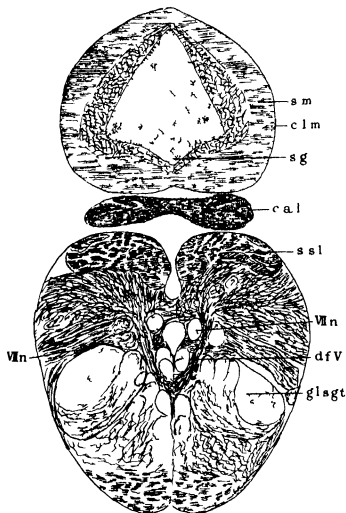


FIG 2—Sole

of the vagals more anteriorly fibres are to be seen passing downwards and outwards from the facial lobes into the great secondary gustatory tracts, these lobes do not extend far in an antero posterior direction neither do they increase in size to any extent laterally. The facial nerves are small

and do not enter the medulla until the tip of the cerebellum appears in section (fig 2) when they pass transversely towards the middle line and then turn caudally and run parallel to the ventricle before entering the

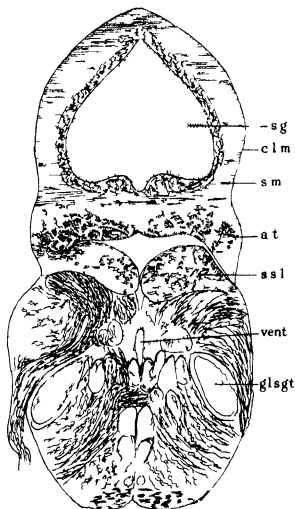


FIG 3—Sole

lobes The somatic sensory lobes completely envelope the facials they extend a long way in the caudo cephalic direction and gradually increase in size anteriorly so as to form the two prominent bulges which are seen in the naked eye view The acoustic lateral areas are large and the size of the lateral line nerves as they enter the posterior crura is remarkable

At the base of the cerebellum, and at first separate from it, is a broad band of tissue roofing the ventricle, on the inferior margin of which are to be seen groups of round cells, with transversely running fibres dividing them (figs. 2, 3), this structure is similar to the areas which I have described in the cyprinoid brain, and, on grounds of comparative anatomy, have associated with an auditory function. This central acoustic lobe is well marked in the herring and in *Mormyrus*, both of which possess elaborate auditory organs, it is noted in the surface-feeding cyprinoids of Europe,

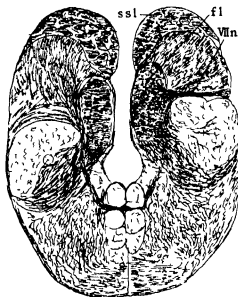


FIG 4—Eel

India, and the great lakes of Africa, but is rudimentary or non-existent in the bottom-feeding species, it also appears to be present in those fish that are known to possess sound-producing organs. The question therefore arises, How are we to account for its presence in the sole?

Before discussing this problem, it seems necessary to give the results of an examination of the medulla oblongata of the eel. Fig 4 is a section across the hinder end of the somatic-sensory lobes of this species, which are of some size. On the right the facial nerve is seen running transversely towards the middle line and ending in an oval-shaped area which produces a slight bulge into the rhomboid fossa, which is very deep and narrow. Below the descending fibres are seen entering the great secondary gustatory



tract in the typical manner. Although the facial lobe does not project on the surface, it is well developed and extends a considerable distance in an antero-posterior direction—approximately 7 mm. There is no central acoustic lobe in the eel, but the acoustic tubercles are large and prominent, as seen in fig 5, their relations to the cerebellum are unique. The dorsal end of each acoustic tubercle bends sharply towards the middle line and joins the cerebellum, the *stratum granulosum* of which is divided into halves and separated by an area of *stratum moleculare*. The result of this

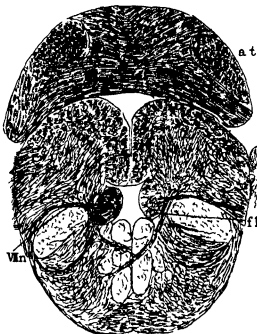


FIG 5—Eel

fusion is to make a V-shaped projection into the cerebellum of tissue composed of coarsely granular substance, with the apex of the V pointing medially (fig 6)

If the cerebellum is traced more anteriorly, it will be noticed that, in contrast with the formation of most teleost brains, it is divided into two by an extension of the ventricle medially, and that this extension ends just above the dorsal margin of the *stratum granulosum* (fig 7)

It will be wise to consider for a moment two important facts in the anatomy of the sole, which have a close relationship with its brain configuration.

The first is the well-known presence of the papillary area on the lower or left side of the head. Bateson (1889) states that "contrary to expectation these villi do not bear sense organs", of the nature of taste buds, and my own independent observations confirm this fact, although it has been denied by Cunningham, but it accords with the evidence, given above, that the facial lobe is very small. We should expect to find it well developed if there were taste buds present in this area. The observation explains also the great size of the somatic-sensory lobe. Bateson also describes the

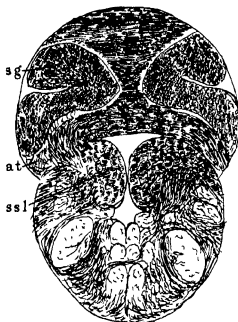


FIG 6—Eel

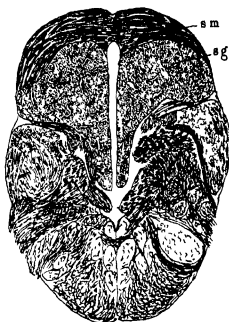


FIG 7—Eel

olfactory organ of the sole "as similar but smaller than those in the conger and the eel. The plates are arranged in two rows on each side of a central raphe upon which the two rows are folded longitudinally so as to form a lining to the olfactory tube. The raphe in the sole is depressed so as to form a groove from which the plates rise up." It is therefore very interesting to find a striking similarity between the olfactory bulbs and lobes of the conger and eel, and of the sole. In the conger, moreover, the right bulb lies partly below the left, much as in the sole. But here the similarity of structure ends, because in *Anguilla* taste buds are present on the tongue and lips, and on the skin of the anterior tubular nostril, while *Conger vulgaris* has taste buds on the outer and inner lips and palate, and

these organs are of two kinds, superficial and deep. Moreover, both of these eels hunt their food with their noses.

As is well known, 'the sole is nocturnal in its habits, and is caught more frequently at night than by day and in the daytime more frequently when the water is cloudy than when it is clear. The sole seeks its food more by sense of smell and touch than by sight, and in the Irish Sea prefers worms (nereids) to any other form of diet' (Jenkins 1925). "According to Cunningham (1896) the sole is distinctly a shoal water fish. The stomachs of thirty six soles were examined and in eighteen the food consisted of marine worms. Small fragments of the shells of bivalves were found, but these seemed in most cases to have been attached to the tubes of tube building worms although small bivalves were sometimes found entire. The throat teeth are pointed and slender and cannot serve for crushing shells as do those of the plaice. Twenty five per cent of the stomachs contained echinoderms mostly sand stars. Crustacea were found in 11%. In soles caught off the west coast of Ireland it was found that worms were most frequent then echinoderms, then molluscs then crustaceans and, lastly, fish in a few cases. The echinoderms were mostly brittle stars, or sand stars among the molluscs were small specimens of the razor shell, the crustaceans were usually small sand hoppers or shrimps, the fish small sand eels." When searching for food the presence of which it recognizes by the smell, the sole glides gently about over the sand tapping with the lower side of the head in order to feel with the sensitive filaments there. The sole is one of the flat fishes most addicted to burying themselves in the sand or gravel leaving only the eyes exposed. It is commonly held by fishermen in the North Sea that soles not only cover themselves when light is directed upon them, but bury themselves during periods of intense cold. Russell states that they take no notice of a worm dangled above them, but Bateson observed that they perceive objects approaching them, for they will bury themselves if a stroke is made with a landing net. He adds that soles, eels and rocklings have a clear appreciation of light and darkness, always being buried during the day and swimming about in the tank at night."

In the description of the brains of the conger and the eel in the physiological series of the *Catalogue of the Royal College of Surgeons* we find. In front of the medulla of *Anguilla vulgaris* the several regions of the brain are of approximately the same size, and each is more or less clearly bilobed. The brain appears to consist of four pairs of rounded equal sized nodules. The anterior pair (olfactory bulbs) are slightly pointed in front and give off two large olfactory nerves. "In the conger eel (*Conger vulgaris*) "the brain is

more elongated than in *Anguilla*. It is remarkable for the large size of its olfactory centres "The olfactory bulbs are of great size and are separated from the cerebrum by short peduncles" (just as in the sole). "Each receives an immense nerve from the olfactory organ. The right bulb lies partly below the left, much as in the sole" "The thalamencephalon is remarkably long for a teleostean, forming a narrow neck between the cerebrum and the moderately developed optic lobes," a description which applies almost equally well to the condition found in the sole

It appears from the above descriptions that the eel type of olfactory organ is associated with a very large olfactory lobe, and it is remarkable that we find this type in only one family of flat-fish. If we consider the diet of a yellow eel and its habits, we find that, according to Tate Regan (1911), it feeds principally at night, its diet is chiefly worms, small fish, cray-fish, etc., although the larger ones are practically omnivorous.

We have now to consider how these habits of feeding and this diet are reflected in the pattern of the medulla oblongata and also in the development of the optic lobes and of the olfactory organs and lobes. In the first place we note that the olfactory system is highly developed in the sole and that it conforms to the type found in *Anguilla* and *Conger vulgaris*. Secondly, the optic lobes are small, as in the eel, and are in marked contrast with the large lobes of the plaice. The somatic-sensory lobes are very prominent and have been mistaken for the facial lobes, but it appears from the study of serial sections that the latter are minute, and this is to be expected, when it is remembered that taste buds are absent not only from the lips and mouth but also from the papillary area on the under-surface of the head. A surprising fact is the presence of a well-marked central acoustic lobe, which hitherto I have noted only in fish that are believed to have a considerable power of hearing. As a general rule, it appears that a central acoustic lobe is well marked when the facial lobe is small, and vice versa. The question arises, why the sole possesses a central acoustic lobe, and I suggest that it is a reasonable assumption to attribute an auditory function to this area, at least we can assume that it is associated with the perception of vibrations. The tapping of the sand, so characteristic of the sole's method of hunting, reminds one of the thrush feeding on our lawns, tapping and listening for the hidden worm. Sea-birds, such as certain gulls and the sheldrake, tap for worms in the same way on our shores. If this conclusion is accepted, it is clear that the sole feeds by smell, touch and hearing, represented centrally in the olfactory, somatic-sensory, and central acoustic lobes, all of which are markedly developed. On the other hand, the eel feeds by smell, taste and touch,

represented centrally in the olfactory, facial and somatic sensory lobes. In both the sole and the eel the habit of night feeding is associated with the small size of the optic lobes.

When we turn to the consideration of group II we notice that the difference in the naked eye appearances of the brains of the sole and the

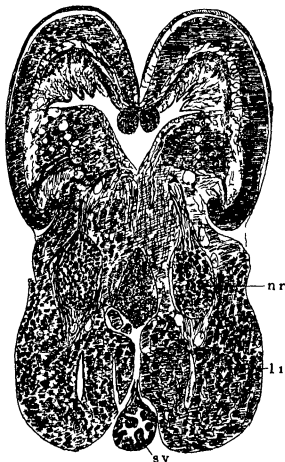


FIG 8—Sole

plaice is much accentuated in the microscopic picture. The two large scale drawings across the middle of the optic lobes (figs 8 9) show the very great prominence of the tectum opticum of the plaice compared with that of the sole. These figures also show the details of the lobi inferiores which will be discussed later in this paper.

The medulla of the plaice is taken as the type of the second group, and

the medulla of the witch will also be described because it presents the peculiar anatomical feature of blister like cavities on the lower side of its head. The chief characteristic of the former is that it is long and narrow the somatic sensory lobes are not prominent as in the sole they meet anteriorly in the middle and posteriorly the rhomboid fossa forms a deep

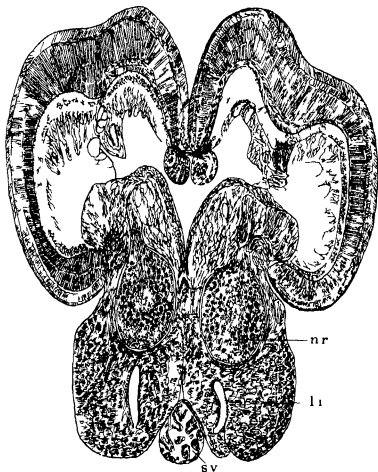


FIG 9—Plaice

cleft. The facial nerves are seen in the drawing (fig 10) to be of considerable size they pass transversely inwards and then turn caudally. The facial lobes are large and form a slight bulge in the walls of the rhomboid fossa they are capped by the fifth lobe and are not evident in a naked eye examination their descending fibres form a strong band passing downwards into the great secondary gustatory tracts. More caudally the nucleus

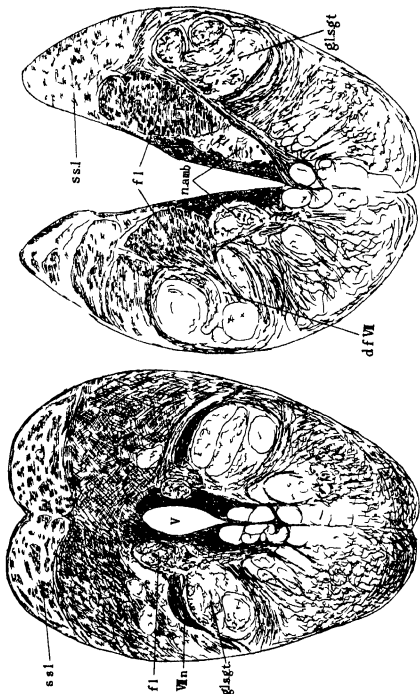


Fig 10—Place

Fig 11—Place

ambiguus appears, but the vagal lobe is of very moderate size (fig. 11). The great difference in size between the facial lobe of the plaice and that of the sole is, no doubt, to be associated with the presence of taste buds in the plaice, which have been described on the palate by Bateson

There is no central acoustic area or lobe. In the witch the somatic-sensory lobe is also narrow and long but projects dorsally, so that it has considerable depth, the facial lobe is very similar to that of the plaice, but is not so large, and produces no bulge in the wall of the rhomboid fossa, and there is no central acoustic area.

Bateson, writing on the olfactory organ of *Pleuronectes*, states that the plaice, flounder, lemon-sole, dab, as well as the halibut (*Hippoglossus vulgaris*), "have only one row of olfactory plates, arranged in a single series in a direction parallel to the long axis of the body and not transversely to it." This is in sharp contrast with the condition that is found in the sole.

Steven (1930), writing on the bottom fauna and the food of fishes, describes the feeding behaviour of the lemon-sole, which depends on eyesight to discover its prey, and forages principally by day. At Plymouth its diet is solely marine worms and annelids. "Tubicolous annelids unless hunted discreetly disappear to safety down their tubes. The lemon-dab is always on the move. It comes to rest in a characteristic attitude with the head and forepart of the body raised well off the bottom. Remaining perfectly still in this position, it scans the ground with its very prominent and movable eyes. Should it then spy a worm cautiously emerging from its burrow, it pounces upon it with a kind of forward leap, bringing its mouth down almost vertically upon its victim by a strong arching of the anterior part of the body." "The plaice and dab behave in a similar manner when searching for food but they do not raise the head quite so high before they pounce."

Cunningham (1896) states that "the plaice feeds chiefly on bivalve shell-fish, whose shells it is easily able to crush by means of the strong blunt teeth in its throat, but it also eats sea-worms. In the Firth of Forth the bivalve most commonly found in the stomachs was a small species called *Scrobicularia*, and next in frequency was the razor-shell, *Solen*, cockles and clams were also present. Of the worms all kinds were devoured—sea-mouse, lug-worms, rag-worms and tube-worms." Sand-stars were sometimes eaten and an occasional shrimp, but fish rarely. "The witch favours marine worms, they were present in 72% of the stomachs examined, Crustacea in 30%, molluscs in 14% and echinoderms in only 3%." "Dabs from the Firth of Forth feed mostly on Crustacea. These occurred in 48% of the stomachs. Next in abundance were echinoderms, namely sand-stars and



brittle-stars, which amounted to 21%. Molluscs were less commonly present, and worms occurred in only 16% of the specimens, sand-eels and small herrings in only 5%. On the other hand, in St Andrew's bay the echinoderms and worms form the staple diet, occurring in 43% of the stomachs."

It will be seen from the above facts that the members of group II are entirely bottom feeders, and the type of brain that characterizes these fish is in accord with their mode of hunting. The olfactory organs are moderately developed, while the optic lobes are very large. The facial lobes are also well marked, but the somatic-sensory lobes are less prominent, as one would expect in fish which are not predatory in their habits and are well provided with taste buds. Considering their habitat, it is not surprising that there is no sign either of an acoustic lobe or central acoustic area. We have not been able to observe specialization of any area in the brain of the witch, which could be associated with the blister-like cavities on the lower side of the head.

#### THE MEDULLA OBLONGATA OF THE TURBOT

The third group is typified by the turbot; serial sections of the brain have been studied both of this fish and the megrim. The medulla of the

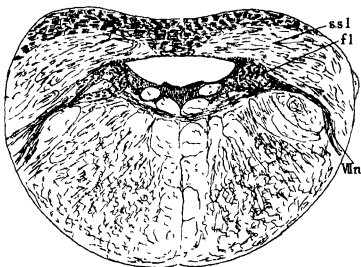


FIG 12.—Turbot

turbot is remarkably broad anteriorly, the somatic-sensory lobes are large (fig 12), and project more prominently as one travels caudally, when they become separated from each other and overlap a somewhat quadrilateral

rhomboid fossa (fig 13), the lateral margins of which bulge slightly through the inward projection of the small facial lobes. The nuclei ambiguus are very marked and the vagal lobes large (figs. 14, 15). In the drawings there will be noted a great thickening of the lining of the ventricle, this is most marked in fig 14. The lower margin is here V-shaped and occupied by a fan-shaped area of tissue. The free edge of this is lined with ciliated epithelium, beneath which are small pear-shaped cells from which fibres pass downwards and then form a fine meshwork. Around the dorsal and lateral margins there are several layers of small round cells. The acoustic tubercles are not prominent and there is no central acoustic area. The cerebellum is

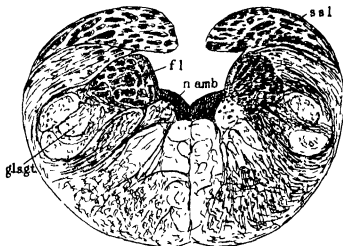


FIG 13—Turbot

characterized by the extent of the stratum granulosum, which occupies the greater part of the tissue, leaving only a narrow margin of stratum moleculare. The medulla of the megrim is not broad, like that of the turbot, but this is compensated by its greater depth. It has a well-marked somatic-sensory lobe and a small facial lobe, which does not project so much into the rhomboid fossa, but we notice the same thickening of the lining of the ventricle as that described above. There are small acoustic tubercles with no central acoustic area. The cerebellum is of the same type as that of the turbot, with a very large area occupied by the stratum granulosum. The features characteristic of this group are the large somatic-sensory lobes and small facial lobes, and the absence of a central acoustic area, whereas the second group had less marked somatic-sensory lobes and well-developed facial lobes.

The food of the turbot according to Cunningham consists almost entirely of other fish. It is a predaceous creature. On the west coast of Ireland the principal fish found in turbot's stomachs were sprats and sand eels, occasionally a dab, a sole or a pout. On the trawling grounds of the

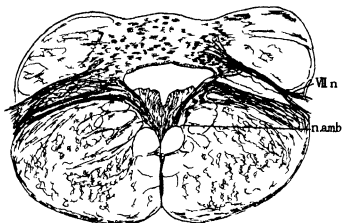


FIG 14—Turbot

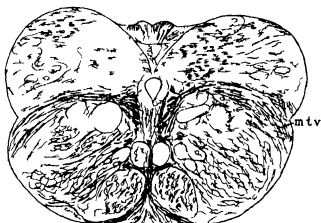


FIG 15—Turbot

south west of England he found in the turbot's stomachs the boar fish known as the cuckoo, pilchards, whiting, sea bream and pout, and never found anything besides fish.

The diet of the megrim, as of the turbot and brill, is fish, namely sprats, sand eels, whiting, gobies, etc. We find here accordingly a group of predaceous fish, and the pattern of the brain is just what would be

expected, being very different in type from that found in group II. The optic lobes are large, the somatic-sensory lobes very prominent, and the facial lobes small—a condition comparable to that found in the pollack, with this difference, that the facial lobe is not apparent, as it is completely concealed by the large somatic-sensory lobes

The brain of the halibut, when examined microscopically, differs little from the preceding group, except in size. The optic lobes are very large, less so in a lateral direction than in depth, and the ventral portion of the tectum opticum bulges so as to give a bilobed appearance. The optic nerves are very large. The olfactory bulb is small and the facial lobe insignificant, while the somatic-sensory lobes are very large. The cerebellum is prominent and its lateral margins embrace these lobes some way posteriorly to a marked extent. There is no central acoustic lobe or area. Other points to be noticed are the prominence of the thalamus and the large nuclei rotundi, which will be described in Part II. The halibut is a predaceous fish, and its diet is mostly fish and crustaceans, as would be expected from the prominent optic lobes and large somatic-sensory lobes.

#### SUMMARY

Our conclusions can be conveniently shown in the form of a table

		Olfactory lobe	Optic lobes	Facial lobes	Central acoustic lobe	Somatic- sensory lobes
<i>Sole.</i>	Night feeder Diet— worms	Large	Small	Very small	Large	Large
<i>Platce.</i>	Ground feeder Diet—worms and shell-fish	Medium	Large	Large	Nil	Medium
<i>Turbot</i>	Diet—mostly fish	Small	Large	Small	Nil	Large
<i>Halibut</i>	Diet—mostly fish and crustaceans	Small	Large	Very small	Nil	Large
<i>Eel.</i>	Night feeder Diet—worms, small fish, etc	Large	Small	Large	Nil	Medium

#### PART II A CONTRIBUTION TO THE ANATOMY OF THE LOBI INFERIORES, INFUNDIBULUM, AND PITUITARY GLAND, WITH SOME REMARKS ON THE BRAIN OF *ANGUILLA*

##### PITUITARY, LOBI INFERIORES AND INFUNDIBULUM

In the monograph on *Pleuronectes* by Johnstone and Cole (1901), the lobi inferiores are described as "a pair of large bean-shaped bodies with their median surfaces in apposition. In the middle line in front of these is the

spherical pituitary body. The apposition of the pituitary body and lobi inferiores is not complete and a triangular space is left by which there emerges on to the ventral surface of the brain the red thin-walled saccus vasculosus.... In the adult this and the pituitary body (hypophysis cerebri) are essentially glandular structures receiving a marked nervous supply from the infundibulum. The third ventricle is prolonged downwards and somewhat backwards into the hollow infundibulum which communicates ventrally with the cavity of the pituitary body, more dorsally it communicates with the large cavities of the lobi inferiores, whilst finally it is prolonged into the saccus vasculosus. Incidentally we may draw attention to the large paired nucleus rotundus "

After a detailed examination of serial sections of this area in plaice, sole, witch, turbot and megrim, it is possible to add some further details of the relations of these closely connected structures. We have found that the series provided by the megrim gave the clearest picture, and drawings (figs (a)-(f), Plate V) will help to make the details clear to the reader. Fig (a) is the most anterior of the series, which has been drawn under a projector. The pituitary gland is not attached, but the depression on the ventral margin of the brain shows the site of its apposition more posteriorly. The layer of small round cells on the margin is to be noted, and especially the group of large multipolar cells lying laterally to the central depression. Dorsally the opening of the third ventricle into the infundibulum is seen. At this level it does not extend more than half-way towards the base, it is lined with ependyma, beneath which is a layer of round cells for the most part, but in the more dorsal part there is a wide area of large multipolar cells, which are shown in a detailed drawing from the sole (fig 20). External to this layer is neuroglia with scattered round cells. The outline of the lobus infundibuli is clearly indicated by its dark staining, and passing ventrally in this tissue are seen small bundles of nerve fibres. Between the lower end of the infundibulum and the margin of the brain is seen a large ovoid bundle of nerve fibres lying transversely, which has been formed by the fusion of two large bundles which pass down from the lateral walls of the infundibulum, to be seen in sections more anteriorly but not shown in this drawing. Fig (b), Plate V, is very similar to the preceding, but the ovoid bundle now appears dumbbell-shaped as it separates into two bundles, these are seen in fig (c), Plate V, where the infundibulum has now extended so as to enter the cavity of pituitary gland, which is here attached laterally by a narrow band of nerve tissue, the fibres from which pass into the small group of multipolar cells situate on the margin, as has already been noted in fig. (a), Plate V; fibres pass from

these along the margin of the thalamus and end in a fine mesh work of fibres and small cells about the level of the large bundles. The gland itself is here seen to have a neurogla portion facing the cavity small in

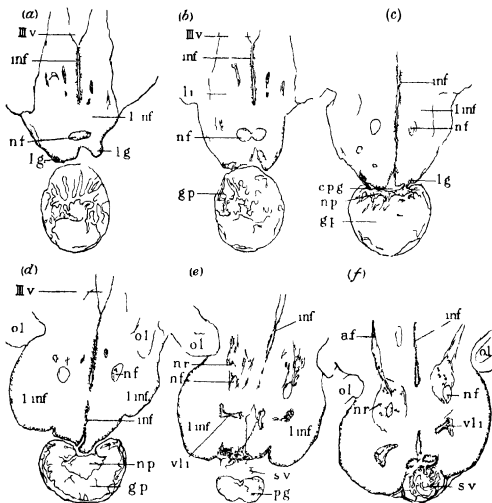


PLATE V.—*Megrim* III v third ventricle nf infundibulum l lobus infundibuli nf nervus lobi infundibuli lg lateral ganglion of pituitary gland np nervous portion of pituitary gland gp glandular portion of pituitary gland ol optic lobe cpg cavity of pituitary gland l inf lobus inferior vl ventricle of lobus inferior sv sacculus vasculosus nr nucleus rotundus af ascending fibres from round nucleus ps pituitary stalk gc ganglion cells of infundibulum apg attachment of pituitary gland pn pars nervosa pi pars intermedia al anterior lobe

area and a large glandular portion into which strands of nerve tissue penetrate. In fig (d) Plate V the lobi inferiores are beginning to appear and in addition to the large lateral bundles smaller bundles are to be seen also some fibres pass from the meshwork we have described towards the main bundles. The gland has now lost its connexion with the brain but the neuroglia portion is encroaching more on the glandular portion. Fig (e) Plate V shows the commencement of the saccus vasculosus lying in the recess between the lobi inferiores and its connexion with the infundibulum. Ventral to it lies the posterior extremity of the pituitary gland. The junction of the cavities of the lobi inferiores with the infundibulum is also shown but the most important feature of this section is the entrance of the large lateral bundles into the nucleus rotundus.

Fig (f) Plate V shows the lateral bundles lying in the centre of each nucleus rotundus into which they merge and ascending bands of fibres pass dorsally from the nuclei and then turn caudally. The saccus vasculosus is larger and the transverse cavity through which it communicates with the infundibulum is now closed. The ventricles of the lobi inferiores are also closed. The minute anatomy of the nuclei rotundi requires special mention on account of the round or oval islets of grey matter which give it a unique appearance these lie in a granular matrix interspersed with nerve fibres. More anteriorly (but this is not apparent at this level) fibres pass from the lobi inferiores into the ventral ends of the nuclei some of these fibres seem to embrace the nuclei but they all pass dorsally into the large strand of fibres passing to the upper part of the thalamus. It therefore appears that the various structures we have been considering are all part of a complex organ in which the specialized tissues which surround the infundibulum are connected by lateral trunks indirectly receiving fibres from the lateral ganglia which as we have seen receive fibres through the lateral attachments of the pituitary gland. All of these nervous connexions are finally carried to the nuclei rotundi which also receive fibres from the lobi inferiores directly. According to the physiological series of the *Catalogue of the Museum of the Royal College of Surgeons* the valvula of the cerebellum is connected by tracts to the lobi inferiores. Schafer and Thain (1893) describing the pituitary body state that the posterior lobe is developed as a hollow outgrowth of the part of that cavity of the embryonic brain which afterwards becomes the third ventricle. In fishes the cells which compose its walls become converted into nerve cells and fibres and as the lobus infundibuli it becomes an integral part of the brain. As to the function of the lobi inferiores we get a little help from Herrick (1905). He states that the ascending secondary gustatory tract of both facial and vagal

lobes terminate in the superior secondary gustatory nucleus, situated in the lateral wall of the isthmus. The chief third gustatory tract arises from the cortical layers of the above. This tract is somewhat obscure, but it has been proved that fibres pass from it ventrally to the lateral lobule of the lobus inferior." He also gives a diagram showing the relations of the olfactory and gustatory paths in teleosts, in which an olfactory conduction path is shown to terminate in the lobus inferior, and adds, "This appears to be the chief centre for the correlation of olfactory and other higher senses."

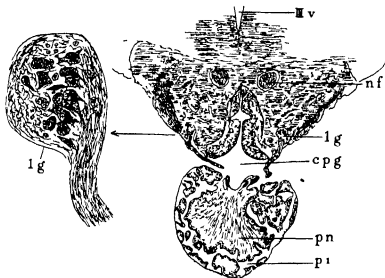


FIG 16

We can now turn to the consideration of the pituitary body and lobus infundibuli in the four groups of Pleuronectidae. The pituitary gland in the various species of Pleuronectidae discloses some marked differences in details of structure. If we take the plaice as a type, we find that the gland is large, and consists mostly of a glandular portion in which the cells are arranged in acini, the margins of which are composed of several layers of round cells. Some sections show that almost the whole gland is made up of this tissue, but there is also a central portion, increasing in size where the gland is attached to the thalamus, and consisting of very fine fibres interspersed with minute cells, which we take to be a specialized neuroglia tissue. This comes to the surface opposite the opening of the infundibulum into the cavity formed by the junction of the gland and the lobus infundibuli. Fig 16 is a drawing of a transverse section, under low power, of the



inferior part of the lobus infundibuli (at the level of fig (c) Plate V) of the plaice. The large pars nervosa consisting of neuroglia tissue is seen penetrating deeply into the glandular portion the pars intermedia. An inset is also shown drawn under high power of the lateral attachment of the pituitary gland containing nerve fibres passing into the cells of the lateral ganglion. The fibres passing from the ganglion are seen passing along the margin of the thalamus to end in a meshwork of small cells and fibres. The two large bundles of the nervus infundibuli are also seen.

#### THE MID BRAIN OF THE PLAICE

Plate VI with diagrams I XII shows the lobi inferiores and the relations of their ventricles to the infundibulum the relations of the latter to the saccus vasculosus and the commencement of the pituitary gland in the plaice. Commencing posteriorly fig I shows the transverse cavity into which the secretion of the saccus vasculosus passes before it enters the infundibulum. II shows the ventricles of the lobi inferiores as they are about to enter the infundibulum. As the sections are followed forward the saccus becomes gradually smaller in V the pituitary gland begins to appear and in VII two portions of this gland are seen in the section and the ventral end of the infundibulum becomes enclosed. Sections VIII XI show the two anterior projections of the pituitary gland becoming larger and the ventral extremity of the infundibulum again approaching the margin finally this opens into a cavity formed by the coalesced margins of the anterior projections of the pituitary and their junction with the inferior margin of the lobus infundibuli. According to Johnstone and Cole (1901) the infundibulum of the plaice is difficult to delimit as it is largely merged into the floor of the thalamus but the above series of sections enables one clearly to visualize the relations of the various cavities to the infundibulum.

#### THE OPTIC THALAMI AND LOBI INFERIORES OF THE PLAICE

The large drawing (fig 9) shows the tectum opticum which is here seen to have a convoluted margin more so on the right of the section than on the left. The saccus vasculosus is seen lying between the opposed lower margins of the lobi inferiores and on either side their ventricles appear. Dorsal to these are the two very prominent ganglia rotunda which have a very characteristic structure round or ovoid areas of grey matter in a meshwork of fine fibres running dorso ventrally in a groundwork of small celled tissue. Their dorsal ends are prolonged towards the cavity of the

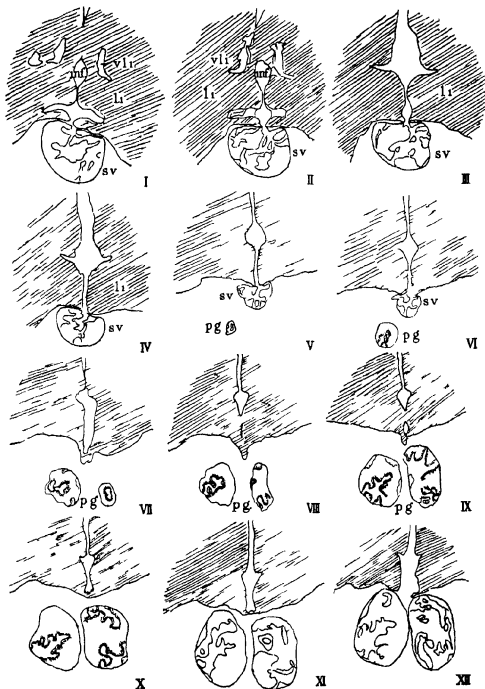


PLATE VI—Plate

optic lobes where they join a band of nerve fibres which turn caudally. The ganglia receive their distal fibres from two sources, fibres enter their lower margin from the lobi inferiores, but also a broad band of fibres can be traced from the centre of either side of the lobus infundibuli and these are shown in the series of outline drawings (figs (a)-(f), Plate V). These have been drawn, by means of a projection apparatus, from sections of the brain of the witch, in which the course of the fibres could be very clearly traced. The connexion of the infundibular lobe with the ganglia rotunda has been also followed in the sole and turbot, in which the course of the fibres are in

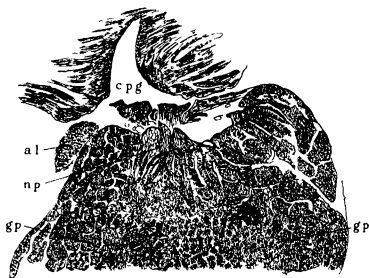
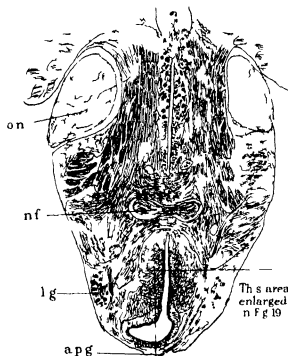


FIG 17—Turbot

all essentials the same. The pituitary gland of the plaice is of moderate size, and consists of a central portion of modified neuroglia surrounded by convoluted tubules lined with several layers of round cells, the so-called *pars intermedia*. The ependyma of the infundibulum is composed of large round cells lying in a marginal area of interlacing fibres. The turbot (fig 17) differs from the plaice in both the general and the minute structure of its pituitary gland. In the first place we notice that the saccus vasculosus is small in the turbot, while the gland is a very prominent structure, equal in size to one of the optic lobes, it is situated farther back than the gland of the plaice. It differs markedly in its microscopic appearance and stains more deeply with haematoxylin, and, as the grouping of its acini is very close, it is hard to define the actual arrangement of the cells, except

towards the periphery and on the margin that faces the thalamus. Areas are to be seen where the masses of cells are breaking down into yellow staining secretion which tends to coalesce and seems to flow towards the nervous portion of the gland which forms the superior portion facing the thalamus. This nervous portion is composed of a meshwork of neuroglia fibres and strands of this tissue pass into the round celled structure which makes up the body of the gland. At the margins of the attachment to the



F1 18—Sole

brain are to be seen small groups of large ganglionic cells similar to those described in the plaice.

Figs 18-22 are drawings of the lobus infundibuli and pituitary gland of the sole and it is at once clear that these structures differ in many points from the conditions found in the plaice and turbot. Fig 18 is the most posterior of the series of transverse sections and shows the closed cavity of the infundibulum lined with small ovoid cells from which a fine process passes outwards into a fine meshwork of nerve fibres. At the apex of the infundibulum the stalk of the pituitary gland has been torn away but a bundle of nerve fibres protrudes through the torn margin which shows the

point of attachment of the stalk of the gland. The fibres passing to the lateral ganglia are well marked and the group of ganglion cells is more prominent than in the other flat-fish which we have described above. The ascending nerves from the ganglia pass directly upwards into a meshwork of small cells and fibres, in the neighbourhood of the dumbbell-shaped band of fibres which form the nervus infundibuli. Fig 19 is a high-power drawing of the lateral ganglion. Fig 20 shows the infundibulum more anteriorly, it is here seen to be lined with several layers of ganglion cells,



FIG. 19—Enlarged drawing of lateral ganglion of sole

the condition being similar to that found in *Anguilla*, as we shall see later. Fig 21 is the lower part of fig 20 under a higher power. Fig 22 is a section of the pituitary gland. The pars nervosa spreads from a central stalk, and its tissue invaginates deeply into the pars intermedia or glandular portion, in the upper left-hand corner is an area which is probably the anterior lobe or the "Hauptlappen" of Stendell (1914). The sole has therefore a stalked pituitary lobe with a very marked nervous connexion, with large lateral ganglia and an infundibulum with a highly developed ganglionic tissue.

Fig 23 is a transverse section of the pituitary gland of the megrim. The pars nervosa is not very extensive in this section, which is an enlarged drawing of fig (c), Plate V. The glandular portion is very similar to that of the turbot. On the left of the section the attachment of the gland to the



FIG 20—s l



FIG 21—Sole

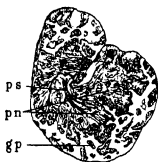


FIG 22—Sole

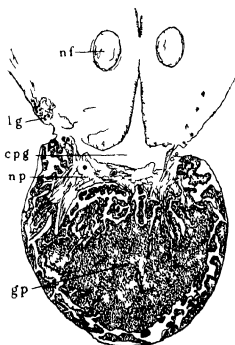


FIG 23—Mogr m

lobus infundibuli is shown, and a well marked lateral ganglion is seen to be present. The two trunks of the nervus lobii infundibuli are large.

Fig. 24 is a transverse section of the gland of the halibut. In general appearance it differs somewhat from the glands of turbot and megrim. The acini of the glandular portion entirely enclose the pars nervosa except for a small gap situated dorsally. The lateral ganglia are well marked but the nerve fibres of the lateral attachment of the gland only appear in fragments. A further point may be mentioned, namely, that the nuclei rotundi are very large.

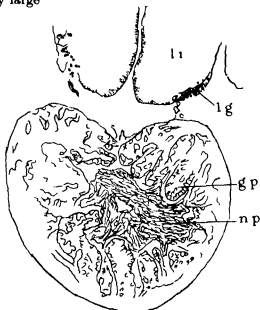


FIG. 24—Halibut

The conclusions that may be drawn from the above observations are, firstly, the presence of lateral ganglia connected by nerve fibres which pass through the lateral attachment to the pituitary gland. This is seen in all the specimens examined. Secondly, the collection of nerve fibres from the lobus infundibuli divides into two large strands which pass backwards on either side of the infundibulum and enter the bilateral nucleus rotundus. Thirdly, there are certain differences in the proportion of the nervous part of the gland to the glandular part, in the plaice group on the one hand, and the turbot and megrim on the other hand, while the sole stands by itself in the structure of its gland and its attachment to the lobus infundibuli, and also in the size of its lateral ganglia. The ganglion cells lying within the cavity of the infundibulum are a striking feature.

The microscopic anatomy of the lobus infundibuli and pituitary body of flat-fish having been considered, we propose to make a few general references to the pituitary gland of teleosts. In the haddock the various parts are not separable (de Beer), but a pars anterior, pars intermedia, pars nervosa, "Uebergangsteil", and saccus vasculosus can be distinguished. de Beer (1926) pictures a longitudinal section of the pituitary of a trout 65 mm long, in which he distinguishes a pars anterior, pars intermedia and pars nervosa. He says "the nervous tissue of the infundibulum appears to penetrate into and excavate the posterior portion of the hypophysis, which here differentiates into the pars intermedia. Anteriorly the tissue contains a number of vesicles."

Stendell (1914) gives a drawing of a sagittal section of the infundibular region of *Anguilla vulgaris*, but does not mention at what period of the

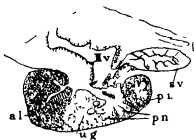


FIG. 25—*Anguilla*, after Stendell



FIG. 26—Ecl

year the specimen was caught. This is redrawn (fig. 25) to make clear the various structures he describes. Anteriorly is the "Hauptlappen" and posteriorly the "Zwischenlappen" or intermediate lobe, and in the centre is the pars nervosa with invaginations of neuroglia tissue which he terms "Hirnteilschlauche". Between this and the anterior portion is a cell area called "Uebergangsteil". Fig. 26 is a drawing of a section of an eel caught in May, and the same areas can be distinguished with the exception of the "Uebergangsteil".

In the course of my examinations of the brain of the Pleuronectidae there appeared certain points of similarity between the brains of the sole and the eel, especially as regards their olfactory organs and lobes, and the small size of their optic lobes, so that an examination of the infundibulum of the sole was naturally followed by similar observations on that of the eel. The large specimen of *Anguilla vulgaris*, from which transverse sections of the infundibulum were prepared, was caught in the autumn during the period of migration, and these specimens show a very remarkable difference from



the pictures of the pituitary gland given by Stendell and from my own drawing from a fish caught in May Fig 27 is a section across the posterior



FIG 27—Pituitary of eel | er od f m gr at or

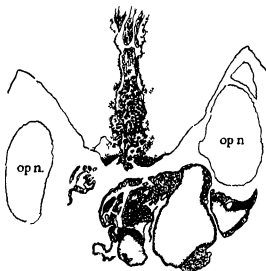


FIG 28

portion and fig 28 across the anterior The large masses of apparently colloid material are very striking and suggest the appearance of an active thyroid gland in a higher vertebrate Surrounding the large areas are

smaller masses of colloid material and also areas of numerous small vesicles in the midst of round celled tissue a stage in the formation of the larger masses The neuroglia portion is not apparent The gland lies opposite the funnel of the infundibulum the margins of which are composed of a mass of large round cells In its lower part the lumen of the infundibulum is composed of several layers of ganglion cells and these are

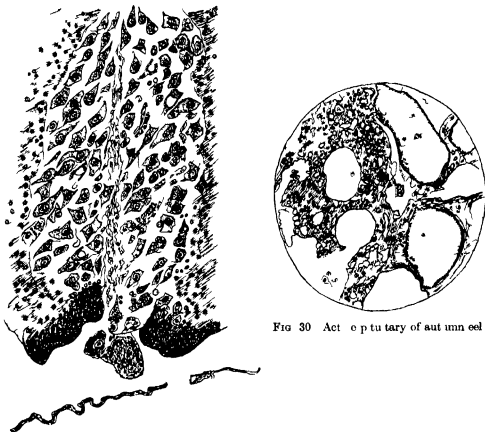


FIG 30 Act e p t u r a r y of a u t u m n e e l

bordered by groups of round cells lying at the margin of the lobus infundibuli (fig 29) Fig 30 is a portion of fig 27 enlarged This condition of the pituitary gland in the eel and its apparent relation to the seasonal changes and the adoption of the marriage garb is an observation which requires further research and confirmation I propose to examine the gland in the summer months at intervals until the autumn

If the above facts are confirmed we shall be in possession of an important

biological key to the profound changes which take place in the external characteristics and in the generative organs of the eel at the period preceding the autumn migration [Note added in proof 16 March 1937 Sections of the pituitary gland of *Anguilla* caught in October and November last show marked changes in the posterior lobe—the changes however are the conversion into granular colloid with small masses of hyaline colloid]

#### SUMMARY

The following conclusions are reached as the result of this research

Part I Four types can be recognized in the brains of the Pleuronectidae

A The sole type with large olfactory lobes small optic lobes a central acoustic lobe and small facial but large somatic sensory lobes The habitat is purely bottom and the feeding is at night The central acoustic lobe is associated with the habit of tapping the sand in search of worms and the smallness of the facial lobes corresponds with the absence of taste buds

B The plaice type with olfactory lobes moderate in size the optic lobes very large no central acoustic lobe but facial lobes well developed Feeding is by sight smell and gustatory sensations They are bottom feeders

C The turbot type with optic lobes well developed the somatic sensory lobes large no central acoustic lobe and small facial lobes These fish are predatory in habit and feed by sight As in those gadoids which are purely fish eaters the somatic sensory lobes are the most developed part of the medulla oblongata

D The halibut type These are also predacious fish and feed by sight The optic lobes are therefore large and in the halibut tend to be convoluted and they extend deeply dorso ventrally The somatic sensory lobes are prominent

Part II The same four types have been studied with regard to the structure of the pituitary body and its relation to the mid brain

Type A The infundibulum is elongated and is lined with well marked ganglion cells The pituitary gland is stalked and the pars nervosa well marked The lateral ganglia are very prominent

Type B The pituitary is of moderate size the pars nervosa well developed and the lateral ganglia well marked

Type C The pituitary gland is very large and the glandular portion very prominent and the anterior lobe is well developed

Type D The lobus infundibuli is very much elongated and the glandular portion well developed and the arrangement of the tubules differs from that in Type C

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## Electrical Activity of the Central Nervous System of the Frog

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(Communicated by A V Hill Sec R S —Received 28 October 1936)

[Plates 13 14]

Careful exploration has been made of the electrical activity of the brain of various mammals (Adrian and Matthews 1934 Gerard Marshall and Saul 1936 Gerard 1936*a* and *b*) but there has been very little study of the brains of other vertebrates (Adrian 1931 Umrath and Umrath 1934) The small size and relatively homogeneous cell population of the Amphibian brain offer certain advantages in analysis of the physicochemical factors determining activity and further the low metabolism of the poikilothermic

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brain at room temperature, together with the short distances for oxygen diffusion, suggested the possibility of studying sustained activity in the isolated organ. The present report covers a general exploration of spontaneous electrical changes in the neuraxis of the grass frog (*Rana pipiens*) and bull frog (*R. catesbeiana*).

### METHOD

The amplifying and recording systems are described elsewhere (Gerard and others 1936). Pick-up electrodes were either of the concentric needle or the bipolar type, in the latter case consisting of either insulated platinum wires or two 32-gauge copper wires, enamelled except for the freshly exposed cross-sections at their tips, and separated 0.5–10 mm. in various experiments. The ground lead was sometimes placed, with the grid lead, on the nervous tissue, sometimes on nearby bone or fascia. Along the spinal cord, especially, the separated leads yielded larger potentials, and these were most commonly used.

Frogs were either decapitated, and the brain isolated from the separated head, or the brain or entire neuraxis exposed under ether and the animal allowed to blow off the anaesthetic. One or two hours are necessary for full recovery from ether, and before this time electrical activity, while present, may be greatly diminished. In occasional experiments curare or strychnine were injected into a lymph sac, but the great bulk were performed on seemingly normal active animals with the brain simply exposed.

### RESULTS

#### *Spontaneous Potentials In situ*

An electrode placed on or in practically any part of the nervous system shows greater electrical restlessness than when controlled on bone, fascia covering muscle, or cotton wool soaked in Ringer solution. This extra activity from the neuraxis disappears under deep narcosis, asphyxia, or some time after "death" of the animal, and may be taken to represent the activity of neurones.

From the *cord and medulla* a rather regular 8 per sec. rhythm was obtained in several experiments. Each wave was sharp, like a spike, but of considerably longer duration, and amplitudes up to 50 or 60  $\mu$ V were observed (fig. 3, Plate 13).

The *optic lobes* manifested considerable spontaneous activity. Several distinct rhythms were present in different preparations or simultaneously

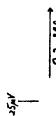


FIG 3



FIG 4

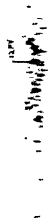


FIG 5



FIG 10

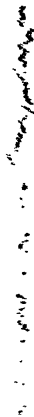


FIG 6



FIG 7



FIG 9

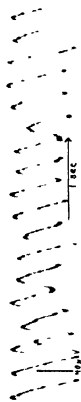


FIG 11



FIG 12



Fig 13

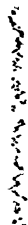


Fig 16



Fig 19

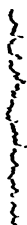


Fig 17



Fig 20

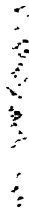


Fig 14

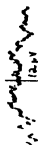


Fig 18

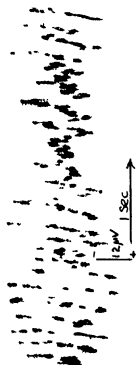


Fig 21



Fig 15

in one. A regular rhythm at 4 or 5 a sec and of 8–50 or more  $\mu\text{V}$  was present in half of the animals. Single waves lasted from 30 to 100 msec. Various other rhythms of greater frequency sometimes appeared, the fastest being irregular at 100–150 a sec with amplitude of 5–10  $\mu\text{V}$  (figs 4–7, Plate 13).

These rhythms were unaffected by illumination of the eyes in some experiments, disturbed in others, but never increased or completely abolished. Most characteristic was a diminution for some time (0.5 sec) after turning the light on, and an increase after turning it off.

The *thalamus* yielded feeble rhythms, not very regular, at 30 a sec and 120–150 a sec with excursions of about 5  $\mu\text{V}$  (fig 8, Plate 13).

The *cerebral hemispheres* were not uniformly active. Quantitatively, the medial surfaces gave distinctly greater potentials, up to 40  $\mu\text{V}$  for the slower rhythm, than other regions, which never exceeded 10  $\mu\text{V}$ . We have not satisfied ourselves that different regions are characterized by qualitatively distinct wave trains—certainly the main rhythm types appeared in all. In only one case, a rhythm (15 a sec) present on the dorsum of a hemisphere was not recorded medially or laterally. As compared to the optic lobe, the electrical profile of the cerebrum is more variable, both in frequency and regularity of rhythm.

A rhythm at 1 or 2 a sec, with maximal potential of 15  $\mu\text{V}$ , was encountered several times on the medial surface, once (4  $\mu\text{V}$ ) on the lateral—possibly as spread from a more active medial region. In one animal large waves of 60  $\mu\text{V}$  and lasting 15 msec appeared rather regularly every 2 sec on the medial surface only.

In every case, a rhythm lying between 4 and 8 a sec (as absolute extremes 3 and 10), most commonly 5, was recorded from the medial hemisphere and, when sufficiently intense, from other portions as well. This was regular in some cases, quite irregular in others, and sometimes an alternation of large and small waves appeared. In various experiments intensity ranged from 5 to 40  $\mu\text{V}$ , and the wave duration up to 0.1 sec. It is possible that, in some cases, the potentials picked up from the hemispheres were spreading from the extremely active olfactory bulbs, but this can be excluded in the majority.

A 15 a sec rhythm, rather regular but in one case beating over a 2 sec period, and with a maximum potential of 30  $\mu\text{V}$ , was most pronounced in the posterior half of the cerebrum, medially and dorsally. Another rhythm, at about 30 a sec and 15  $\mu\text{V}$  amplitude, was encountered also on the medial surface, from a more anterior point. Finally, irregular discharges, from 50–100 a sec, in one case even 200, were commonly present, with intensities up to 10  $\mu\text{V}$  (control about 2  $\mu\text{V}$ ). In some brains a fairly constant rate, of 50,



or 65 or 80 a sec was maintained in others no such restraint existed These discharges were to some extent produced by electrode injury but not solely for they were present without or long after penetration of the brain tissue

Almost without exception when several rhythms were simultaneously present amplitude diminished as frequency increased This would naturally result if cells were discharging more in phase at the lower rates less at the higher A similar consideration predicts greater amplitude with greater regularity at any given frequency This is particularly well illustrated in the olfactory bulb

The *olfactory bulb* is in terms of potentials dramatically outstanding in the nervous system in the frog (figs 11 and 12 Plate 13) Intact or *in vitro* its electrical activity dominates the picture as the visual system appears to dominate in the cortex of man In every case greater potentials were obtained from this structure than anywhere else A similar situation exists in the fish (Hoagland personal communication) The fundamental activity is a rhythm at 4 a sec 3-8 in extreme cases often extremely regular and of over 80  $\mu$ V swing at the maximum Average potentials were about 20  $\mu$ V Individual waves sometimes occupied the full interval of 0.25 sec sometimes lasted only 0.1 sec in the former case leading from the surface the rising limb (positive) lasted 80 msec the falling 160 while from a deeper position the rise was longer than the fall Occasionally a rhythm waxed and waned in a regular beat with a 2-3 sec period and amplitude fluctuation from maximum to only one fourth maximum with evidence such as double peaks or humps in a wave of two rhythms making beats In one case a double rhythm was clearly present with a total frequency of 9 a sec

Still slower changes appeared in two cases spike like waves of 20  $\mu$ V at irregular second intervals and a 2 a sec rhythm of 10  $\mu$ V which showed beats The 15 a sec rhythm (20  $\mu$ V) appeared only once in an isolated brain The 25-30 a sec rhythm was commonly present ordinarily of 5-10  $\mu$ V intensity In one case leading coronally from the surfaces of both bulbs a very regular rhythm at 25 a sec and 18  $\mu$ V was present Other rapid oscillations at frequencies from 40-100 a sec and rather irregular were observed in about half the experiments These were usually about 5  $\mu$ V in amplitude sometimes 10 and in one case with a rather regular rate of 70 a sec 20  $\mu$ V In two cases 25  $\mu$ V spikes at intervals or 10  $\mu$ V ones at 35 a sec disappeared a few minutes after placing the electrodes and are attributable to injury or stimulation

With both leads on one or both olfactory nerves no potentials above 6  $\mu$ V were recorded *In situ* these appeared at 25-30 a sec in one isolated

brain irregularly at 50-100. Since the only approaches to the olfactory bulb—the olfactory nerve ahead and the hemisphere behind—show far less activity than the bulb itself, the bulb activity is presumably due to a "spontaneous" beat of the neurones in this structure and is not dependent on the advent of nerve impulses. The final evidence on this point comes from the electrical activity of isolated brains.

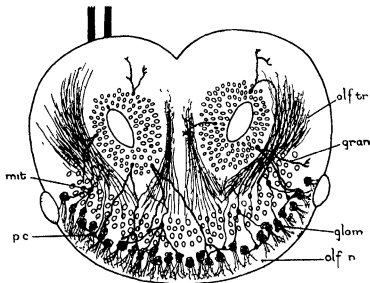


FIG. 1

FIGS. 1 and 2—Diagrams of structure and probable cell connexions in the olfactory bulb and cerebral hemispheres of the frog. The figures follow especially the accounts of Ramon (1896), Rothug (1926) and Herrick (1910, 1924), and all the cell-types and fibre tracts figured have been described by histologists, though it must be emphasized that the connexions of each type of cell are not yet fully known. *glom*, glomerulus, *gran*, granule cell, *lfb*, lateral fore-brain bundle, *mfb*, medial fore brain bundle, *olf tr*, olfactory tracts, *olf n*, fibres of olfactory nerve, *p c*, periglomerular cell, *mit*, mitral cell. The electrodes are shown as they would be placed for recording potentials from the olfactory bulb and from the dorso-medial surface of the cerebral hemisphere.

*In vitro*. Isolation of hemispheres and olfactory bulbs, with or without the thalamus and optic lobes, was carried out in a number of cases after exploration *in situ*. The brain stem was cut across, cranial nerves severed, membranes loosened or removed, and the isolated brain lifted away on to cotton wool soaked in Ringer solution. The same types and almost the same magnitudes of potentials were obtained as from the intact brain, and they commonly became larger, slower, and more regular with increasing time after

isolation for at least 90-120 min. An irregular 7 a sec rhythm, varying from 4-10  $\mu$ V, obtained from the optic lobe soon after isolation, had become regular at 4 a sec and 12  $\mu$ V 1½ hr later. One from the olfactory bulb at

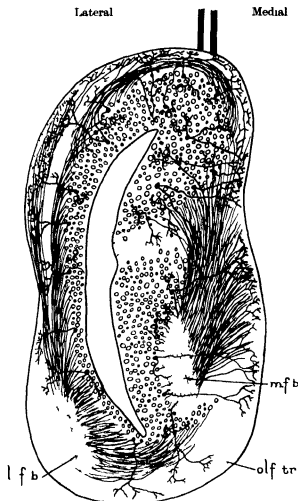


FIG 2

6 a sec was 20  $\mu$ V after isolation and placing the electrodes, 35  $\mu$ V when left undisturbed for 3 min. Another from the bulb was irregular from 4-8 a sec and 8-16  $\mu$ V after isolation, very regular at 6 a sec and 15  $\mu$ V 90 min later and with no manipulation during the preceding 10 min.

## DISCUSSION

It seems clear from these results on the olfactory bulb that neurones are able to beat electrically in the absence of afferent nerve impulses bringing to them some needed excitation, and in the absence of spurious stimulation or injury by the conditions of the experiment. That these potentials are the result of the spontaneous activity of the cells and are not due to local tampering is well shown by the fact that both in intact and in isolated brains the rhythm often increased in amplitude and regularity after the electrodes had been in place for some time, and was not greatest immediately after placing. Neurones in general may possess the property of automaticity, as do those of the heart and the respiratory centre (Shown most clearly by the continued potentials, at the rate of normal respiration, in the isolated insect or fish brain (Adrian and Buytendijk 1931, see also Prosser 1934)). The rate and amplitude of the rhythm of a single cell must be determined by physico-chemical factors in itself or environment. The magnitude and character of the potentials led from a mass of neurones in some gross division of the brain will be determined, in addition to the activity of single cells, by the sequence and synchrony of discharge of the whole cell population. When, as in the olfactory bulb or other grey masses, a smooth regular wave of constant rate and amplitude appears, the presumption is strong that the bulk of cells are acting in unison. If so, the recorded wave shape would be that of the single cell potential. We incline to the view that this is so in some of our experiments, and hope this preparation will be favourable for study of cell potentials. This view is in harmony with Adrian's first position (Adrian 1931) rather than his second one (Adrian and Matthews 1934) which attributes all slow waves to summed spikes. We have found at least, in contrast to the experience of Adrian and Matthews on the mammalian cortex, that large slow potentials are readily recorded from electrodes within a millimetre of one another or even concentric.

It is noticeable that the observed potentials did not parallel motor effects. For instance, placing of the electrodes in the mid-brain regularly caused very active movements of various parts of the animal, but the potentials recorded from this region were much smaller than those from the olfactory bulb whose injury or stimulation is not usually followed by any movements at all.

For correlating potentials and structure in the fore-brain of the frog the following anatomical facts are significant (see Ramon 1896, Herrick 1910, Kappers 1921). Although many architectural regions can be distinguished (Imogawa 1935), the cells and their connexions are broadly similar

throughout the entire thalamus, cerebral hemispheres and olfactory bulb. Even the pallial and subpallial part of the hemispheres do not differ greatly, there being no true cortical layers.

The characteristic pyramidal cell (see figs 1 and 2), usually lying close to the ventricle, has a dendrite which spreads into the peripheral neuropil and an axon which, after sending collaterals to the interstitial neuropil surrounding the cells, runs either to some other part of the hemisphere or to more caudal brain parts. The mitral cells of the olfactory bulb may be considered as modified pyramidal cells which have migrated away from the ventricle.

An ample opportunity for interaction between neighbouring pyramidal cells is provided by common connexions in the peripheral neuropil, by the axon collaterals surrounding the cells themselves (interstitial neuropil, Herrick 1934), and by intercalated small cells whose irregularly distributed short processes probably serve to integrate the activities of the pyramidal cells. These small cells are especially developed in the olfactory bulb as the granule cells, which lack axons but have two dendrites both ending among those of the mitral cells. There is, therefore, an adequate structural basis throughout the telencephalon, and especially in the olfactory bulb for the co-ordinated action of large groups of cells.

The latero-dorsal region of the hemisphere (primordium pyriformis, Herrick) which is reached by many axons of the mitral cells of the very active olfactory bulb is less active than the medio-dorsal region (primordium hippocampi) which is reached by few, if any. It is, of course, under strong olfactory influence through additional connexions from in front and below. Intrinsically the medial wall is much thicker than the lateral, but whereas the cells of both pallial and subpallial parts of the lateral wall are closely packed around the ventricle, those in the medial wall are spread through its whole thickness, with a particularly dense interstitial neuropil occupying the spaces between them. If the neuropil helps to synchronize cell potentials, recorded activity should be more regular and stronger medially than laterally. Our findings are in harmony with this expectation but do not lead to a definite conclusion as to the function of the neuropil.

#### SUMMARY

The exposed or isolated neuraxis of the frog has been explored for spontaneous electrical activity.

Spontaneous potentials independent of injury or motor effects were noted throughout the central nervous system. Various regions were more or less

characterized by the rate, regularity and intensity of their potential profiles, though three distinct frequencies might be present in one place at different times or simultaneously

The olfactory bulb was in all cases electrically dominant, with a rhythm often extremely regular at 4 a sec and up to 80  $\mu$ V intensity. This, and other spontaneous rhythms, persisted for 2 hr or more with the brain isolated on moist cotton wool, and often improved in regularity and amplitude for much of this time.

The findings are considered in relation to anatomical structure and especially the neuropil. It is concluded that neurones are able to manifest a rhythmic electrical beat in the absence of afferent nerve impulses or local injury.

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## DESCRIPTION OF PLATES

Plate 13 All figures are of spontaneous rhythms from brains exposed and left *in situ*

FIG. 3—Lower spinal cord. Grid on left of cord, 1 mm. anterior to ground, which is on right.

FIG. 4\*—Optic lobe, surface. Time in this and all subsequent figures as in fig. 11.

FIG. 5\*—Same as 4, but electrodes penetrating.

FIG. 6\*—Optic lobes, electrodes transversely across midline. Just after placing electrodes. Amplification shown on 4.

FIG. 7—Optic lobe, electrodes in sagittal plane.

FIG. 8—Thalamus, same as 7. Electrodes  $\frac{1}{2}$  mm. off midline. Record same as control on moist cotton. Amplification shown on 7.

\* These observations on the same bull frog.

FIG. 9—Cerebrum, mid-dorsal position, soon after deep insertion of electrodes. Injury discharge.

FIG. 10\*—Control on moist cotton. Amplification shown on 11.

FIG. 11\*—Olfactory bulb. Transverse electrode position.

FIG. 12\*—Same as 11, later. Note beat as if units get into and out of phase.

#### Plate 14. Isolated brain activity

FIG. 13—Control on moist cotton.

FIG. 14—Olfactory bulb, exposed in decapitate head. Amplification shown on 13.

FIG. 15—Same, 10 min. later. Preparation and leads undisturbed. Note increased activity. Amplification shown on 13.

FIG. 16—Same, 90 min. later. Leads replaced. Amplification shown on 18.

FIG. 17—Optic lobe, taken just after 16. Amplification shown on 18.

FIG. 18—Control on moist cotton, just after 17.

FIG. 19—Optic lobe, freshly isolated brain. Amplification shown on 21.

FIG. 20—Olfactory bulb surface, immediately after 19. Amplification shown on 21.

FIG. 21—Same as 20, 3 min. later. Nothing disturbed. Note larger and more regular potentials.

\* These observations on the same bull frog.

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## Studies on Codehydrogenases I—Nature of Growth Factor "V"

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*(Communicated by D. Keilin, F.R.S.—Received 12 November 1936)*

In 1917, Davis recognized that two growth factors are required by influenza bacilli. A few years later, Thjotta and Avery (1920, 1921) were able to confirm Davis's data and named these substances "X" and "V" factors respectively. The "X" factor is actually well known chemically (Ghon and Preys 1902-4, Davis 1917, etc.) and physiologically (Lwoff, A. 1936a). The so-called "growth factor V" has been studied by numerous workers (Davis 1917, 1921, Fildes 1921, Thjotta and Avery 1920, 1921; Rivers and Poole 1921, Thjotta 1924, and others) but its nature is still unknown.

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"Growth factor V" is found in yeast, blood corpuscles, and animal and vegetable tissues. It is water soluble, sensitive to heat, being destroyed by heating in alkaline but not in acid solutions. It passes through Berkefeld filters and collodion membranes, and is partially adsorbed by bone charcoal. It is destroyed by contact with fresh serum of certain animals in 4 hr at 37° C, the destructive power of which can be removed by heating at 70° C. It is further known that vitamin C is unable to act as "V" factor.

It does not seem necessary to extend this introduction. An historical account and the properties of the "V" factor are to be found in Scott's article (1929), in the papers of Anderson (1931) and of Meyer (1934) and in the more recent book of Knight (1936). We may say briefly that nothing is actually known about the nature and physiological function of the "V" factor, which are to be discussed in this paper (see preliminary paper Lwoff, A. and M. 1936).

#### MATERIAL AND METHODS

For physiological tests of factor "V" we have used *Haemophilus parainfluenzae*, strain 4101, of the National Collection of type cultures, Lister Institute—isolated by V. Fleming (London). We obtained the strain through the courtesy of Dr. St. John Brooks and Miss Rhodes, to whom we are glad to express our gratitude.

The culture medium had the following constitution: proteose peptone Difco 20 g, sodium chloride 6 g, distilled water 1000 ml, NaOH to bring it to pH = 7.5. In this medium the multiplication of *H. parainfluenzae* can only take place if "V" is added. The best source of "V" is yeast, as pointed out by Thjotta and Avery (1920, 1921). The yeast extract is prepared as follows: 1 kg of baker's yeast is emulsified in distilled water and the emulsion is brought to pH 4.5 by addition of hydrochloric acid. This emulsion is poured gently into warm distilled water (80° C) so that the temperature never falls below 75° C. It is then brought to 80° C and kept at that temperature for 20 min. During the heating, the pH is kept constant by the addition, if necessary, of hydrochloric acid. The hot emulsion is filtered through a Buchner funnel and the clear yellow liquid thus obtained is filtered through a Chamberland candle  $L_3$ . The extract obtained, when 1 l of water has been utilized for 1 kg of yeast, is active at a dilution of 1/50,000.

In the subcultures a solution diluted to 1/10 may be used—one drop of which (with a pipette giving 30 drops/ml) added to 9 ml of culture medium allows a very good growth.



For the experiments the subcultures are made with one drop of a culture grown in a medium containing yeast extract 1/5000. In these conditions the first subculture is always negative if the new medium is devoid of "V" factor. Nevertheless, in each of our experiments we always subcultured a control tube without "V" which, without exception, always proved negative

When making dilutions of "V" solution we always used washed and sterilized pipettes (2 hr 160° C), so that every possibility of bringing in "V" factor with the pipettes is completely excluded. The cultures are incubated at 37° C.

#### FRACTIONATION OF YEAST EXTRACT—WARBURG'S COENZYME AND COZYMASE

Yeast extract was treated with different reagents and the subsequent fractions tested qualitatively and then quantitatively for their "V" activity

We could see that the "growth factor V"

- (1) is not precipitated by barium acetate at pH 8.5,
- (2) is not precipitated by lead acetate at pH 6.8,
- (3) is precipitated by lead acetate at pH 9.5,
- (4) is precipitated by mercuric nitrate at pH 7.0,
- (5) is precipitated by alcohol (final concentration 90%),
- (6) resists desiccation *in vacuo*

At this point in our work we discussed the problem of "V" with Dr T. Mann, who called our attention to the fact that all the above properties of "V" factor were common to those of cozymase. We had at the time no cozymase but only a preparation of pyridine nucleotide triphosphate (Warburg and Christian's coenzyme of the hexosemonophosphate oxidation system). This pyridine nucleotide triphosphate, extracted from horse's blood corpuscles, had been obtained some months before from Professor Warburg.

The initial degree of purity was 0.3, but the substance, originally a white powder, had not been kept *in vacuo* and had turned into a viscous brown substance. This substance proved to be active in dilution of 1/30 millions. Taking into consideration the degree of purity of this substance, it may be seen that the preparation was active in dilution of 1/100 millions. But we may assume that the substance had absorbed water and perhaps lost a part of its activity and that the limit of active dilution must be much higher.

The question then arose whether or not the action of pyridine nucleotide triphosphate was specific and if it could be replaced by pyridine nucleotide diphosphate.

Dr D. E. Green provided us with a solution of yeast cozymase, the approximate degree of purity of which was 0.7 and the approximate concentration M/500. This solution proved to be active as "V" factor at a dilution, calculated for the dry weight of cozymase—of 1/270 millions. It must be added that this cozymase preparation—as tested by D. E. Green—is completely devoid of any trace of pyridine nucleotide triphosphate; it possesses no activity when tested with hexosemonophosphoric acid as substrate.

The limits of dilution we give are of course not absolute magnitudes. When approaching limits of dilution, the action of "V" factor is quantitative, that is to say there is a relation between the amount of "V" and the number of bacteria. We took as a limit a quantity sufficient to produce a bacterial turbidity easily seen. But nevertheless the order of magnitude of the limit of dilution of codehydrogenase acting as growth factor "V" is to be found around 1/250 and 1/350 millions.

The quantitative study of blood reinforces this conclusion. Rabbit blood was studied. Caution must be taken in such experiments, as it is well known that serum and blood corpuscles stroma are able to destroy the "V" factor after haemolysis. We proceeded in three different ways: (1) the blood, obtained by heart puncture and defibrinated, is immediately haemolysed (1 part of blood + 9 parts of distilled water) and immediately diluted in the culture medium, (2) the blood is inactivated as soon as haemolysis has taken place, (3) the blood is inactivated immediately after defibrination and then haemolysed and diluted. There is no noticeable difference in the results obtained with blood treated by these three methods if the blood is diluted very quickly.

The active limit of blood dilution is 1/3000, but it is possible to distinguish a very slight bacterial turbidity at 1/4000 and sometimes at 1/5000. It is known (Warburg, Christian and Griese 1935) that the horse-blood concentration in pyridine nucleotide triphosphate is 1/150,000. If we arbitrarily assume that the rabbit-blood content is approximately the same, we can say that the blood coenzyme acts at a dilution of 1/450 millions. But the blood contains also cozymase in a still unknown amount. The activity of blood as "V" factor must be considered as the sum of activity of cozymase and pyridine adenosine triphosphate. It may be seen, therefore, that the order of magnitude of blood dilution in relation to the concentration in codehydrogenases corresponds roughly to the figures which have been found with purified cozymase.

This activity of codehydrogenases at dilutions of 1/250 to 1/300 millions could be considered as very strong evidence that growth factor "V" is a codehydrogenase (see note, p. 359).

We shall find additional proofs of this fact in studying (1) the properties of reduced "V" factor, and (2) its physiological functions.

#### OXIDIZED AND REDUCED "V" FACTOR

As already pointed out, it was shown by Fildes (1921) that "V" factor withstands boiling in acid solutions but is very rapidly destroyed by boiling in slightly alkaline solutions. Codehydrogenases possess the same property. But reduced codehydrogenase (we shall use here the term "reduced" in the sense of reversibly reduced only) possesses inverse properties and is heat-stable in alkaline solutions. It was interesting to ascertain whether the "V" activity of codehydrogenases was following the same rule. We could see that (1) solutions of oxidized pyridine nucleotide di- and triphosphate keep their "V" activity after heating for 40 min. at 100° C. at pH 4.2, and (2) the "V" activity is completely destroyed after 20 min. at 100° C. at pH 8.5.

If the codehydrogenases are reduced, the "V" activity is not destroyed by heating in alkaline solutions. 10 ml. of each of the two codehydrogenase solutions were reduced by adding 2 ml. of the following solutions, prepared just before using: anhydrous  $\text{Na}_2\text{S}_2\text{O}_4$  0.1 g., phosphate buffer M/10 pH 7.3 10 ml. The solutions of reduced codehydrogenases are brought up to pH 9.5 by addition of NaOH N and heated in a water bath at 100° C. for 40 min. These solutions after neutralization and filtration proved to be still active as "V" factor.

The activity having in this case not been tested quantitatively, it is not possible to say whether there was partial destruction or not. Nevertheless, it can be pointed out as certain that the "V" activity of oxidized codehydrogenases is completely destroyed by heating at 100° C. for 20 min. at pH 8.5, and that the "V" activity of reduced codehydrogenases is not—or only partly—destroyed in 40 min. at 100° C. at pH 9.5.

#### SPECIFICITY OF CODEHYDROGENASES

It has been shown by Euler and Myrbäck (1931-35) that Harden and Young's cozymase contains adenylic acid and by Warburg and Christian that the active group of both codehydrogenases was the  $\beta$ -nicotinamide,

Harden and Young's cozymase being a pyridine nucleotide diphosphate and Warburg's coenzyme a pyridine nucleotide triphosphate. The result of previously described experiments is that growth factor "V" must be considered as equal to codehydrogenases.

The conclusion is therefore that *H. parainfluenzae* is unable to synthesize codehydrogenases, that is to say pyridine nucleotide phosphate compounds. This disability may be due to the fact that the synthesis of one of the components of codehydrogenase is impossible. To see whether this hypothesis was correct or not we tried the activity as growth factor of the different parts of codehydrogenases. The solutions of the substances were sterilized by filtration and studied at dilutions from 1/10,000 to 1/200 millions. For each substance a control of non-toxicity was made to two tubes of medium containing the maximum amount of experimental substance "V" factor was added and growth took place normally.

Yeast and muscle adenylic acid, nicotinic acid, its amide\* and diethylamide were tested and proved to be unable to act as growth factor "V" (see note, p. 359).

We may therefore conclude that the need of growth factor "V", that is to say codehydrogenases, is due to the lack of power of synthesizing the compound adenine + pentose + phosphoric acid +  $\beta$ -nicotinamide, the exact mode of grouping of which is still unknown.

It has been established by parallel study of the morphological evolution of protozoa and of their power of synthesis that a "physiological evolution" must be considered which is characterized by a decrease in the power of synthesis, that is to say, a loss of function (Lwoff 1932). Similar conclusions were arrived at independently by Knight (1936) considering the power of synthesis of bacteria. The problem of growth factors has been specially studied from this point of view (Lwoff 1936 a, b) and it was shown (1) that all the chemically known growth factors are fundamental and general constituents of living organisms, (2) that this need of growth factors is secondary—being due to a loss of power of synthesis and not to new growth requirements.

If we consider codehydrogenases as growth factors, we see that

- (1) Codehydrogenases are to be found in all living tissues
- (2) All free-living and parasitic bacteria, with a very few exceptions, synthesize codehydrogenases, being able to act as nursing bacteria for *H. parainfluenzae*
- (3) The absence of ability to synthesize codehydrogenases is therefore secondary and due to the loss of this specific ability

\* Synthetic preparation of D. Hartree

## BIOCHEMICAL APPLICATION

0.004% of codehydrogenase added to 1 c.c. of peptone solution allows the growth of *H. parainfluenzae*, and therefore this must be considered as the most sensitive test of pyridine nucleotide phosphates

It is very important, when this test is performed, to work at pH 7.5. In presence of excess of "V" the growth is excellent at pH 6.8 and 7.5. But in presence of a small amount of "V" good growth can be obtained at 7.5, though no noticeable growth takes place at pH 6.8. This will be discussed in the following paper. It is highly advisable when these tests are performed

- (1) To add glucose to the media (after sterilization)
- (2) To add (after sterilization) phosphate buffer M/1 pH 7.5, to bring the final concentration to M/20
- (3) To use as inoculum a 24 hr. old culture grown with a very small amount of "V", so that the control without codehydrogenase should be negative

Under these conditions the result is obtained in 12-18 hr. (37° C.)

## SUMMARY

Growth factor "V" from yeast (extract obtained at 80° C. at pH 4.5) is not precipitated by barium acetate at pH 8.5 nor by lead acetate at pH 6.8. It is precipitated by lead acetate at pH 9.5, by mercuric nitrate at pH 7.0 and by alcohol. It withstands desiccation *in vacuo*.

Growth factor "V" can be replaced by purified codehydrogenases (see note, p. 359). Both Harden and Young's cozymase and Warburg's coenzyme are active.

The "V" activity of codehydrogenases which is destroyed in 20 min. at 100° C. at pH 8.5 remains unchanged at pH 4.2 for 40 min. at 100° C. On the other hand, the "V" activity of codehydrogenases reduced with sodium hydrosulphite can be shown even after heating 40 min. at 100° C. at pH 9.5.

When approaching the active limit of dilution of "V" the bacterial growth is closely related to the amount of the growth factor. The maximum dilution compatible with the development of a bacterial cloudiness visible to the naked eye is about 1/270,000,000 (dry weight of codehydrogenase).

The amount of growth factor necessary for growth at pH 6.8 is greater than at pH 7.5.

Adenylic acid (yeast or muscle), nicotinic acid, nicotinic acid amide and diethylamide are unable to act as growth factors

*H. parainfluenzae* and some other organisms which need "V" for their growth are to be considered as having lost the power of synthesizing codehydrogenases, that is to say, pyridine nucleotide phosphates

It is possible by means of a growth test with *H. parainfluenzae* to detect 0.004  $\gamma$  of codehydrogenase

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[Note added in proof 16 February 1937—Thanks to the courtesy of Professor Otto Warburg, we were able to test the activity of new preparations of pyridine nucleotide di- and triphosphates. The preparations, according to Professor Warburg are "fast rein, enthalten aber noch eine kleine Menge der fluoreszierenden Begleitsubstanz, wahrscheinlich para-dihydro-nucleotid"]

The two pyridine nucleotides were active as "V" factor, the limit of the activity of pyridine nucleotide triphosphate being about 1/600,000,000.

On the other hand, we are able to add that the orthodihydropropyl nicotinamide, kindly given to us by Dr F Stare, is devoid of "V" activity.]

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## Studies on Codehydrogenases

### II—Physiological Function of Growth Factor "V"

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#### HISTORICAL

Despite the facts that nothing was known about the nature of growth factor "V" and that no experiments had been performed in order to test its action, some hypotheses have been built up with regard to its possible physiological function for influenza bacilli. These hypotheses have been reviewed by Scott, L. Anderson and M. Pittman.

#### INTRODUCTION

The fact having been established that codehydrogenases are necessary for the growth of *Haemophilus parainfluenzae* the problem of their physiological function for this organism still awaited solution (See preliminary paper A. and M. Lwoff 1936)

The working hypothesis was that codehydrogenases are used as such by the bacteria. As one of the functions of codehydrogenases is to transfer activated hydrogen from the substrate-dehydrogenase to a hydrogen acceptor, we expected to be able to detect the function of codehydrogenases in studying their effect on the speed of reduction of methylene blue and on the oxygen uptake by bacteria.

It must be pointed out that as *H. parainfluenzae* does not grow without codehydrogenase, codehydrogenases must be present in the growing cells. As a matter of fact, when studying bacteria grown with an excess of "V" (15 hr. old culture grown after heavy inoculation in 100 ml. of peptone + 3 ml. of pure yeast extract) we were not able to detect any action of cozymase on the speed of reduction of methylene blue or on the respiration. The culture medium and the bacteria being saturated with growth factor "V" it is quite natural that the addition of more codehydrogenase should be without effect.

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In order to study the specific effect of codehydrogenases it is necessary to use the method already described (A. Lwoff 1933, 1934) in the study on the physiological action of haematin as a growth factor for "parahaemotrophic" organisms.

This method consists in growing the organism with a small amount of growth factor so that the amount of growth factor is the only limiting factor of the development. For *H. parainfluenzae* we added to 200 ml of peptone solution (in a flat flask 20 cm. diameter) 1.5 ml of yeast extract diluted 1/10. With this concentration the density of the culture was approximately 1/6 of the density obtained with an excess of yeast extract. The inoculum of the flasks consisted of 1 ml of a 24 hr old culture grown in the presence of yeast extract 1/5000. Under these conditions the bacterial growth is almost entirely finished after 20 hr.

The bacteria of the culture grown under these conditions will be referred to as culture or bacteria "V-". The culture and bacteria grown with excess of "V" will be called "V+."

#### CONDITION OF THE EXPERIMENTS

To measure the reduction time of methylene blue, 5 ml of a "V-" culture were introduced into a Thunberg-Keilin tube. As growth can take place if codehydrogenase is added, the time of reduction under this condition must therefore be small, approximately 10 min.

The best procedure to study the oxidation of various substrates is to centrifuge off the culture, to suspend the bacteria in Ringer-phosphate pH 7.5, M/20, to centrifuge off again and to resuspend them in a fresh buffer solution. Bacteria treated in this way will be referred to as washed bacteria.

If, for example, we measure the time of reduction of 50  $\gamma$  of methylene blue by a 26 hr old "V-" culture, grown in presence of glucose\* we find it to be 80 min without added cozymase, and 8 min when cozymase is added.

The ratio 
$$\frac{\text{time of reduction without codehydrogenase}}{\text{time of reduction with codehydrogenase}}$$
 will be referred to as "index of codehydrogenase activation" or "i c a." Several conditions must be realized in order to obtain consistent figures of i c a.

(1) An excess of codehydrogenase is necessary and 5  $\gamma$  per ml of bacteria suspension is convenient for this purpose. As will be shown later, when a small amount of codehydrogenase is added the speed of reduction varies with the added amount.

\* Initial pH 7.5, final pH 6.8, phosphate buffer M/1, pH 7.5 is added in the Thunberg tube to bring it to concentration M/20.



(2) It is necessary to keep the tube 7 min. at 38° C. before adding the methylene blue from the side bulb, because an incubation period is necessary to allow the codehydrogenases to act within the cells.

*Example*—"V—" bacteria grown in presence of glucose are washed, 5 ml. of the suspension are introduced into Thunberg-Keilin tubes. Codehydrogenase is added either to the bacterial suspension or to the methylene blue in the side bulb. After 7 min. at 38° C, methylene blue or methylene blue + codehydrogenase is mixed with the bacterial suspension. The reduction time is as follows (a) bacteria without codehydrogenase, 55 min., (b) codehydrogenase in the suspension, 82 sec., (c) codehydrogenase in the side bulb, 255 sec. Incubation period  $255 - 82 = 173$  sec. In other experiments the incubation period when either cozymase or Warburg's coenzyme were added (glucose or pyruvate being used as substrate for both codehydrogenases) was between 90 and 165 sec. In some cases the incubation period was found to be 3, 5 and 6 min. The average period of incubation was found to be  $2\frac{1}{2}$  min. Nevertheless, before adding methylene blue to the Thunberg tube from the side bulb in all our experiments the tubes are kept 7 min. at 38° C.

(3) The reaction of the liquid is very important. In the presence of an excess of codehydrogenase the speed of reduction of methylene blue is approximately the same at pH 6.8 and 7.5 (1/10 slower at 6.8). But when no codehydrogenase is added to "V—" bacteria the speed of reduction is 5-7 times slower at pH 6.8 than at pH 7.5. This is probably due to a more rapid destruction of codehydrogenase at pH 6.8.

This has been verified by growth experiments.

(a) If an excess of "V" is added, very good growth is obtained at pH 6.8 and 7.5.

(b) In the presence of a small amount of "V" the growth is slow and the final density of the culture is less at 6.8 than at 7.5.

(c) It is possible to find a concentration of "V" at which good growth takes place at 7.5 and no growth at 6.8 (for these experiments the peptone solutions are buffered after sterilization with sterile phosphate M/1 to bring them to a concentration M/20).

(d) It must be noticed that when "V—" bacteria are kept at room temperature the i c a may increase. It is therefore necessary to compare only figures obtained with the same emulsion at the same time.

#### CHANGES OF I C A DURING THE CULTURE

We may assume that the value of i c a gives a measure of the codehydrogenase content of the cell. If the i c a is, for example, 2, this would mean that 50% of the codehydrogenase is missing. The percentage of destroyed codehydrogenase would be given by the formula 
$$\frac{(i c a - 1) \times 100}{i c a}.$$

It must be noticed that, according to this equation, small changes in the value of  $i c a$  between 1 and 2 correspond to important differences in the percentage of destroyed codehydrogenase

It is interesting to follow the value of  $i c a$  during the whole development of a culture. During the first phase of the growth the  $i c a$  was 1. Sixteen hours after the inoculation the growth seemed to cease almost completely, showing an increase of only  $1/36$ th per hour during the following few hours.\* At this moment the  $i c a$  was 1.53. That is to say, 30 % of the amount of cozymase was destroyed. The case recorded above is of a culture grown without glucose. The  $i c a$  given is for cozymase measured by comparing the speed of reduction of methylene blue in the culture itself, with and without cozymase. In the following hours the  $i c a$  increased as follows: 16.5 hr 1.53, 18 hr 2.64, 20 hr 5.75, 22 hr 13.6

In this case, the percentage of destroyed cozymase was 30 % per hour of the actual amount of cozymase present in the cell. The only thing we want to emphasize here is that when 26 hr old "V—" cultures, grown under the conditions described, are studied the  $i c a$  varies between 7 and 30, that is to say, 85–97 % of the codehydrogenase has been destroyed. A 26 hr old "V—" culture thus provides us with good material for the study of the physiological function of the codehydrogenase.

#### OXIDATION OF VARIOUS SUBSTANCES BY "V—" BACTERIA

"V—" bacteria (grown in presence of glucose) are carefully washed so that the controls without substrate, with or without cozymase, show no reduction of methylene blue. These bacteria have been tested for their ability to oxidize various substrates and the results obtained are summarized in Table I. The speeds of reduction are averages of two or three measurements.

It is seen that in presence of cozymase (1γ/ml of suspension) glucose is oxidized much more rapidly than other substrates, but in absence of cozymase lactate and succinate are oxidized quicker than glucose. This proves that the lactic and succinic dehydrogenase of *H. parainfluenzae* are able to work without codehydrogenase, like the yeast lactic dehydrogenase. The slight increase in speed of reduction of methylene blue when cozymase is added must be connected with the fact that the oxidation of pyruvate and fumarate (oxidation products of lactate and succinate) depends on codehydrogenase.

\* The age of the culture depends, of course, on the amount of fluid inoculated, on the amount and physiological conditions of inoculum and the amount of "V" factor present.

TABLE I

The speed of reduction of a given amount of methylene blue. When glucose is used as a substrate in presence of an excess of codehydrogenase the speed is arbitrarily taken as 100

If  $T$  is the time of reduction of methylene blue in presence of glucose + codehydrogenase,  $t'$  the time of reduction for another substrate, the speed for this substrate is  $\frac{T \times 100}{t'}$

	Speed of reduction of methylene blue by washed "V - " bacteria grown in peptone + glucose	
	Without added codehydrogenase	+ added codehydrogenase (in Thunberg tube)
0 (control)	0	0
Glucose	3	100
Pyruvate	< 0 15	9
Fumarate	< 0 15	5 5
Malate	< 0 15	5
Ethyl alcohol	< 0 15	7
Asparagine	< 0 15	5
Valine ( <i>d</i> and <i>l</i> -)	< 0 15	2 3
<i>d</i> -Lactate	13	17
Succinate	6	13 5

We must add that, despite the fact that lactate is oxidized without codehydrogenase, *H. parainfluenzae* is unable to grow in peptone + lactate when no codehydrogenase is added

#### ACTION OF CODEHYDROGENASES ON RESPIRATION AND GLYCOLYSIS

The increase in respiration of washed "V - " bacteria, grown in peptone + glucose and suspended in Ringer phosphate + glucose, when cozymase is added is 9. This is the average of three measurements of oxygen uptake in presence of KOH. When the respiration is measured, in order to obtain a sufficient oxygen uptake without cozymase, it is necessary to take suspensions five times thicker than those with cozymase. Otherwise, the oxygen uptake would be too small.

Table II shows the effect of cozymase on respiration and aerobic and anaerobic glycolysis, measured with Warburg's "Kästchen" method. It is seen that increase of both aerobic and anaerobic glycolysis are approximately twice the increase of respiration and that the increase of glycolysis is of the same order of magnitude as the increase of respiration. Other experiments gave a relative value of respiration to glycolysis as follows:  $X_{O_2} : 100$ ,  $X_M^{O_2} : 79$  and  $X_{O_2} : 100$ ,  $X_M^{N_2} : 180$ . The  $Q_{O_2}$  has not yet been determined.

TABLE II

Metabolism of bacteria grown in peptone + glucose (Warburg's "Kästchen" method)  
 Washed bacteria suspended in Ringer bicarbonate + glucose Gas mixture 5% CO<sub>2</sub>/O<sub>2</sub>  
 or 5% CO<sub>2</sub>/N<sub>2</sub>.

	$X_{O_2}$	$\Delta X_{O_2}$	$X_M^{O_2}$	$\Delta X_M^{O_2}$	$X_M^N$	$\Delta X_M^N$	Meyerhof's quotient
Without cozymase	8.7		3.9		7.8		0.45
		11.5		20.4		19.3	
With cozymase	100		81.5		150		0.68

80% of the respiration is inhibited by HCN M/500 or M/1000 when the substrate is peptone, lactate or succinate, but only 45–50% when glucose is oxidized. *H. parainfluenzae* contains cytochrome and catalase.

## QUANTITATIVE ACTION OF COZYMASE

As the action of cozymase on the growth is quantitative, it was expected that its action on the rate of methylene blue reduction would also be quantitative and this would enable us to obtain a figure of the "turn over" ("Wechselzahl"). Experiments were done in the following way:

"V—" bacteria grown in presence of peptone + glucose were washed and suspended in Ringer phosphate. Glucose was added and the reduction time of methylene blue measured in presence of various amounts of cozymase. As the control without cozymase is able to reduce methylene blue, a correction is necessary so that the figure should represent the speed of reduction of methylene blue due to the added cozymase only. If  $T$  is the time of reduction of the control without cozymase,  $t'$  the time of reduction with cozymase,  $Tt'/T - t'^*$  represents the time of reduction of methylene blue connected with added cozymase (Table III). A definite relation exists between the amount of added cozymase and the speed of reduction of methylene blue. The discrepancy between the data obtained and those theoretically expected is rather high, but so many factors are involved in these experiments that it is difficult to expect better results and these results are quite sufficient to give an idea of the order of magnitude of the turn over.

\* In the time  $t'$  the control (without added cozymase) has reduced the  $t'/T$  part of methylene blue.

In the period  $t'$  the added cozymase is then responsible for the reduction  $1 - (t'/T)$  part of methylene blue.

To reduce the total amount of methylene blue a time  $x = \frac{1}{1 - (t'/T)} \times t' = Tt'/(T - t')$  would be necessary.

TABLE III

Added cozymase	Time of reduction of 100γ of methylene blue (in sec)	$Tt'/T-t'$	Theoretically expected	No. of mol. of methylene blue reduced in 1 sec. by 1 mol. of added cozymase
0	1540 = $T$	—	—	—
0 0166	1234 = $t'$	6850	13200	1 62
0 166	588 = $t'$	940	1320	1 29
1 66	122 = $t'$	132*	132	0 9
16 6	61 = $t'$	—	—	—
166	60 = $t'$	—	—	—
0	2090 = $T$	—	—	—
0 0332	1740 = $t'$	10400	5250	0 7
0 1668	930 = $t'$	1678	1050	0 7
1 668	100 = $t'$	105*	105	1 15
16 68	75 = $t'$	—	—	—
Average				1 06

\* Time of reduction with 1 66γ cozymase taken as comparison number

The turn over in this case is the number of molecules of methylene blue reduced in 1 sec at 38° C by 1 mol of *added* cozymase. When the turn over is calculated by measuring the increase of oxygen uptake due to a given amount of cozymase, the result (Table IV) is approximately the same. In this case, the turn over for added cozymase is twice the number of oxygen molecules taken up in 1 sec †

TABLE IV

Added cozymase in γ	Oxygen up- take = $X_{O_2}$	$X_{O_2} - 6 2$	Theoretically expected	Turn-over (for 1 sec)
0	6 2 mm <sup>3</sup>	0	—	—
0 1668	10 3	4 1	4 1	0 96
1 66	40 2	34	41	0 8
33 36	50 4	44 2	—	—

The turn over found, approximately 1 by the two methods, is relatively low when compared with 56, the figure found by Warburg and Christian for the "Wechselzahl" of pyridine nucleotide triphosphate, working with the hexosemonophosphoric system. But it must be taken into account that the turn-over value is calculated in our case by assuming that all the added cozymase is quantitatively absorbed and quantitatively involved.

† We may add that the number of molecules of methylene blue reduced by a given bacterial suspension in presence of glucose and cozymase is twice the number of molecules of oxygen taken up by the same suspension at the same time.

in the oxidation of glucose, and that this is probably not the case. If we measured the turn over in presence of pyruvate as substrate, we should find a much lower value. The value given must therefore not be considered as normal.

#### BIOCHEMICAL APPLICATION

By means of the growth test 0.004  $\gamma$  of codehydrogenase may be easily detected. By means of the methylene blue reduction test 0.016  $\gamma$  has proved necessary when working with 5 ml of bacterial suspension. It would certainly be very easy to work out a micro-method which would allow detection of 0.001  $\gamma$  of codehydrogenase in approximately 20 min.

#### THE SPECIFIC FUNCTIONAL UTILIZATION

"V -" bacteria grown in peptone + glucose are unable to oxidize glucose. But "V -" bacteria grown in peptone alone without glucose are still able to oxidize glucose. If the age of the culture is conveniently chosen, it is possible to find bacteria (Table V) which oxidize peptone very slowly and oxidize glucose at the same speed, with or without added codehydrogenase. (The speed of reduction of culture + codehydrogenase and culture + glucose + codehydrogenase is the same.)

TABLE V

Culture "V -"	Age hr	Reduction time of methylene blue by 5 ml cultures				i c a W's cof	% de- stroyed cozy- mase*	% de- stroyed W's cof.*
		+ 0	+ glucose	glucose + code- hydro- genase	i c a cozy- mase			
+ glucose	24	180	180	12	15	15	93	93
+ 0	26	38	10	10	3.8	1	74	0
+ 0	48	390	42	8	42	5.2	98	81

\* % destroyed codehydrogenase =  $\frac{(i c a - 1) \times 100}{i c a}$  W's cof = Warburg's co-ferment (pyridine nucleotide triphosphate)

In order to explain these facts, a number of experiments were made, the results of which are given below co-ordinated with others already mentioned.

(1) "V -" bacteria grown in peptone + glucose are unable to oxidize pyruvate, fumarate, malate, alcohol, asparagine, valine

(2) "V—" bacteria grown in peptone alone are unable to oxidize pyruvate and fumarate (other substrates not tested).

(3) "V—" bacteria grown in peptone alone or peptone + pyruvate are able to oxidize glucose and hexosemonophosphate (Robison's ester)

(4) "V—" bacteria grown in peptone + glucose are unable to oxidize glucose and hexosemonophosphate (Table VI)

TABLE VI

"V—" bacteria grown in peptone	Reduction time of methylene blue by 5 ml culture (in min.)					i c a Warburg's cof
	+0	+ glucose	+ hexose-mono-phosphate	+ codehy-drogenase	i c a cozymase	
+0	330	19	25	15	22	15
+ Pyruvate	> 90	12	12	12	> 8	1
+ Fumarate	90	9	—	9	10	1
+ Glucose	100	96	92	20	5	43
+ Hexosemono-phosphate	75	75	75	10	75	75

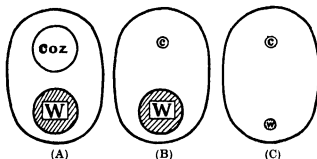


FIG. 1—(A) Bacteria grown with excess of "V" = bacteria "V+" (B) and (C) Bacteria grown with small amount of "V" (V limiting factor = "V—" bacteria) (B) In peptone alone (C) In peptone + glucose or + hexosemonophosphate Coz = cozymase (pyridine nucleotide diphosphate) W = Warburg's coenzyme (pyridine nucleotide triphosphate)

(5) "V—" bacteria grown in peptone + hexosemonophosphate are unable to oxidize glucose and hexosemonophosphate

The following conclusions may be drawn from these experiments.

(a) The codehydrogenase connected with the oxidation of pyruvate, fumarate, malate, and alcohol is cozymase. It therefore appears that cozymase is used up in "V—" bacteria grown in peptone (Exps. 1 and 2).

(b) The oxidation of glucose and hexosemonophosphate is not connected with cozymase (Exp. 3).

(c) Codehydrogenase connected with glucose and hexosemonophosphate oxidation is used up when bacteria are grown in presence of glucose or hexosemonophosphate (fig 1) (Exps 4 and 5)

(d) As the codehydrogenase of hexosemonophosphate oxidation is Warburg's coenzyme, in "V-" bacteria grown in peptone alone or peptone + pyruvate Warburg's coenzyme is not used up (Exp 3)

(e) Warburg's coenzyme is used up in presence of its specific substrate only (Exps. 3 and 5)

(f) The oxidation of glucose is connected with the same codehydrogenase as the one of hexosemonophosphate oxidation (Exps 3, 4 and 5) This is good evidence that one method for the breakdown of glucose by *H parainfluenzae* is preceded by a phosphorylation, the phosphorylated sugar being then oxidized by a system in which Warburg's coenzyme acts as codehydrogenase It would be difficult to explain otherwise why Warburg's coenzyme is used up in presence of glucose, except by admitting that this coenzyme could be connected with the oxidation of another substrate than hexosemonophosphate (Table VII)

TABLE VII

Substrate	"V-" bacteria grown in peptone			
	Alone or + pyruvate	+ glucose or hexosemono- phosphate	If codehydro- genase is added (in the Thunberg)	Codehydro- genase in- volved in the oxidation
Glucose	+	0	+	Warburg's coenzyme
Hexosemonophosphate	+	0	+	"
Pyruvate	0	0	+	Cozymase
Fumarate	0	0	+	"
Malate	0	0	+	"
Ethyl alcohol	0	0	+	"
Asparagine	0	0	+	"
Valine (d- and l-)	0	0	+	"
"Peptone"	0	0	+	"
Succinate	+	+	+	None
Lactate	+	+	+	"

+ = rapid reduction of methylene blue    0 = slow reduction of methylene blue.

(g) Cozymase is used up in bacteria grown in peptone alone. We have seen that methylene blue is reduced by washed "V-" bacteria at a measurable speed when amino-acids are given as substrates only when codehydrogenase is added The speed of reduction in this case is of the same order of magnitude as the speed for fumarate and malate We



have probably to deal here with the oxidation of the non-nitrogenous part of amino-acids. As peptone contains amino-acids the using up of cozymase in peptone may be connected, at least partially, with the oxidation of the products of breakdown of the amino-acids.

(h) The question was considered as to whether the decrease of codehydrogenase might not be due to a diffusion outside the cell or to a special enzymatic destruction, however Warburg's coenzyme is used up in presence of its specific substrate only, and the rate of destruction of cozymase in "V -" bacteria is 30 % per hour of the actual content. For this reason it seems probable that the using up of codehydrogenases is intimately connected with their function: they disappear only when they act and probably because they act. It must be considered that this destruction has been detected only when growth has ceased, because growth takes place only when codehydrogenases are present in the culture media. This using up could of course not be detected with bacteria synthesizing codehydrogenases.

As was shown by Warburg and co-workers pyridine nucleotide phosphates can be over-reduced by nascent hydrogen in presence of platinum black, this over-reduction being irreversible (the pyridine ring being converted into a piperidine ring). On the other hand, Warburg found in blood corpuscles a pyridine compound supposed to be a "wrongly dehydrogenated" pyridine compound. It is possible, though only hypothetical, that the destruction of codehydrogenase connected with their action might be due either to over- or to para-reduction.

#### FREE AND COMBINED CODEHYDROGENASE

(a) It has been shown that both pyridine nucleotide di- and triphosphate enable "V -" bacteria to oxidize glucose and pyruvate and that they both allow the growth in peptone solution. It therefore appears (fig. 2) that the following reaction, pyridine nucleotide diphosphate  $\rightleftharpoons$  pyridine nucleotide triphosphate may occur within the cell. The incubation period being very small, 90-150 sec., this reaction is very rapid.

(b) But we are obliged to conclude that bacteria may exist (Tables V, VI and VII, and fig. 1 B) which contain a "normal" maximum amount of Warburg's coenzyme and a very small amount of cozymase. Warburg's coenzyme disappears very slowly in bacteria grown in peptone alone. In 48 hr. old "V -" culture, that is to say, in the conditions of our experiments, the bacteria, having not divided for 28 hr., still contain 20 % of the normal amount of Warburg's coenzyme (Table V). From this it may be concluded

that bacteria are unable to convert Warburg's coenzyme into cozymase or that this reaction is exceedingly slow.

This conclusion is in complete contradiction with the conclusions of paragraph (a), but as the experiments on which these two conclusions are based are presumed to be correct, and as the reasoning is presumed to be correct too, explanation must be found for this contradiction. Everything becomes clear if we admit the hypothesis that codehydrogenase may exist in the cells in two states (fig. 2)

(1) A free state immediately following the absorption, a free state in which the reaction pyridine nucleotide diphosphate  $\rightleftharpoons$  pyridine nucleotide triphosphate is possible

(2) A combined state in which this reaction is no longer possible

According to Theorell the study of the migration of coenzyme and dehydrogenase in an electric field gives no indication of any compound formation between the enzyme and the coenzyme

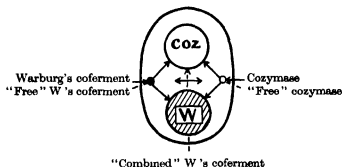


FIG. 2—Diagram showing the relationship between "free" and "combined" pyridine nucleotide phosphates

But the fact that enzymes may exist in the cell in a combined stage is well known especially for amylase (Oparin 1934). Moreover, according to Euler and Myrbäck, dried washed yeast, freed from cozymase, is unable to ferment during 6 hr but after this period fermentation may take place. In this case, we are dealing with combined, inactive enzymes which can be recovered only after some hours. In the case of *H. parainfluenzae* we have a combined active Warburg's coenzyme, the combination of which does not prevent its activity but makes impossible its transformation into cozymase.

It seems therefore that the only way to explain our results is to admit that codehydrogenase may exist in the cell either in a free or in a combined

state and that probably the combined state is between dehydrogenase and its codehydrogenase. This conclusion is based upon the study of the physiology of codehydrogenases within the cells.

We are deeply indebted to Professor D. Keilin for the kind hospitality given in his Institute and for many helpful suggestions throughout the course of this work. We wish to thank Dr. Tadeusz Mann for his assistance during the isolation of "V" and most particularly for his valuable suggestion as to the actual nature of the growth factor.

#### SUMMARY

(1) Codehydrogenases are without action on the speed of reduction of methylene blue and on the oxygen uptake of bacteria grown in presence of an excess of "V" (= bacteria "V +")

(2) Codehydrogenases increase the speed of reduction of methylene blue by bacteria grown with a small amount of "V", the amount of "V" being the only limiting factor of the growth (= bacteria "V -")

(3) The ratio of time of reduction of methylene blue without codehydrogenase to time of reduction with codehydrogenase, or index of codehydrogenase activation (= i c a), varies from 1 to 100 according to the age of the culture.

(4) An incubation period of 90–150 sec. is necessary to allow the codehydrogenases to act in the cell.

(5) The oxygen uptake of "V -" bacteria of i c a 25 is increased about ten times by codehydrogenases. The aerobic and anaerobic glycolysis about twenty.

(6) The action of codehydrogenases on the speed of reduction of methylene blue and of respiration (when approaching the limit of dilution) is quantitative. The turn-over number—number of molecules of hydrogen transferred in 1 sec. by 1 mol. of added codehydrogenase—is approximately 1.

(7) "V -" bacteria grown in peptone + glucose are hardly able to oxidize glucose, hexosemonophosphate (Robison's ester), pyruvate, fumarate, malate, ethyl alcohol, asparagine, *D*- and *L*-valine. All these substrates are rapidly oxidized if cozymase or Warburg's coenzyme are added (i c a  $\geq$  30).

(8) In "V -" bacteria grown in peptone alone or peptone + pyruvate, cozymase is used up.

(9) In "V -" bacteria grown in peptone alone or in peptone + pyruvate, Warburg's coenzyme is not used up. These bacteria are able to oxidize

glucose and hexosemonophosphate at the same speed, with or without added codehydrogenase.

(10) In "V-" bacteria, grown in peptone + glucose or peptone + hexosemonophosphate, Warburg's coenzyme is used up

(11) Warburg's coenzyme is used up in presence of its specific substrate only.

(12) The reaction pyridine nucleotide diphosphate  $\rightleftharpoons$  pyridine nucleotide triphosphate is possible within the bacteria when codehydrogenases are supplied

(13) Pyridine nucleotide triphosphate may exist in the bacteria in a combined state, being potentially active as a coenzyme of the oxidation system of hexosemonophosphate and being unable to undergo conversion into pyridine nucleotide diphosphate

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## The Induction of Ovulation in the Rabbit, by Electrical Stimulation of the Hypothalamo- hypophysial Mechanism

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[Plates 15–17]

In two recent papers, by Marshall and Verney (1936), and by Harris (1936), the mechanism concerned with ovulation in the rabbit has been fully discussed. In view of this, only a brief summary of the present position will be given here.

Ovulation in the rabbit occurs normally only after some form of sexual excitement. There is much evidence to show that the factors involved are first, a nervous stimulation from the genital region and perhaps from the cortex, acting on the anterior lobe of the pituitary gland, and secondly, a hormonal factor, the pituitary gland secreting a gonadotropic hormone which affects the ovaries.

The nervous pathway by which the pituitary gland is stimulated has not yet been fully determined. Cajal (1894) was the first to show that the posterior lobe of this gland is in direct nervous connexion with the hypothalamus. More recently, Greving (1925, 1926) and Pines (1925) have shown that the supra-optic and paraventricular nuclei supply the posterior lobe with fine non-medullated nerve fibres passing through the infundibulum. The only known nerve supply of the anterior lobe is from the superior cervical ganglion, through the carotid plexus (Dandy 1913). No fibres have been traced from the pars nervosa to the pars anterior.

The three main hypotheses which have been put forward concerning the pathways of nervous impulses to the anterior lobe of the pituitary are as follows.

(1) That the impulses pass along sympathetic fibres in the superior cervical ganglion and the carotid plexus. These are the only nerves which have been found entering this lobe of the gland. That this is not the only path has been shown by Vogt (1931, 1933), Hinsey and Markee (1933),

and Brooks (1935), who have all found that rabbits ovulate in a perfectly normal manner after extirpation of the superior cervical ganglia. On the other hand, Haterius (1933) showed that sympathectomized rats failed to become pseudo-pregnant following artificial stimulation, whilst Friedgood and Pincus (1935) managed to obtain ovulation in rabbits after electrical stimulation of the superior cervical ganglia, a result that Haterius (1934) had previously failed to obtain.

There is therefore evidence that the cervical sympathetic system plays some part in this mechanism, but presumably not a very large part.

(2) That there are sympathetic fibres from the central nervous system which supply the pituitary gland, other than those passing through the superior cervical ganglion. Thus Hinsey and Markee (1933) suggest that stimuli may pass to the anterior lobe, via the greater superficial petrosal nerve and the carotid plexus, that is, along paths described by Cobb and Finesinger (1932) and by Chocobski and Penfield (1932).

There appears to have been no experimental evidence put forward for or against this theory.

(3) The third possibility is that there occurs a humoral or nervous transmission of stimuli from the posterior to the anterior lobe of the pituitary, the posterior lobe itself being affected by nerve fibres from the hypothalamus. The evidence for this has been mainly of a negative character, concerning the effect of hypothalamic and pituitary-stalk lesions, on the sex cycles of various animals. Camus and Roussy (1920) showed that, in dogs, damage of the hypothalamus performed with a hot needle led to genital atrophy, though the pituitary gland was left intact by the operation. Bailey and Bremer (1921) found that genital atrophy in dogs followed lesions to the tuber cinereum performed by the temporal route so that there was no danger of concurrent damage to the pituitary gland. Smith (1926) found that lesions of the tuber cinereum in rats produced, amongst other effects, genital atrophy. Cushing (1932*a*), in discussing Smith's results, says that "in all probability this gonadal effect is merely another instance of interference with hypophysial blood supply or of interrupted nerve supply". Richter (1934) cut the stalk, also in rats, and obtained great prolongation of the oestrous cycle.

This evidence certainly seems to suggest that the hypothalamus and stalk of the pituitary gland are concerned with sexual activity, probably as a functional unit, together with the anterior lobe.

The purpose of the present work was to discover whether the hypothalamus and stalk of the pituitary gland played any part in the mechanism underlying ovulation in the rabbit. Three types of experiments

were performed to find the effects on the ovaries of; first, lesions of the stalk; secondly, electrical stimulation of the pituitary gland directly; and thirdly, electrical stimulation of the hypothalamus.

#### LESIONS OF THE STALK

The original object of these experiments was to see whether rabbits would still ovulate following copulation, after section or lesions of the stalk.

The operative technique was developed in collaboration with Professor G. T. Popa, for whose invaluable aid and advice I am greatly indebted. A paper will shortly be published describing the surgery in full, so that it will suffice for the present to mention that the temporal route was used, that this included removal of the posterior half of one zygomatic arch and the superior half of the ramus of the mandible. The lesions were made with a small piece of razor blade, which was ground to the required shape and mounted at the end of a fine probe. It should be mentioned that the diaphragma sellae is well developed in the rabbit, so that during suprasellar operations there is no danger of incidental injuries to the pituitary gland.

Many rabbits died in fits within 2-3 days of the operation. It is believed that this was partly due to meningitis (the condition for asepsis being poor), and partly due to injury of the hypothalamus and tuber. (See Bailey and Bremer 1921.)

Eventually six rabbits, four does and two bucks, were obtained, with lesions of varying extent in the stalk. Three showed transient polyuria for a few days following the operation, but a quick recovery to normal occurred in each case.

The following symptoms were the same in all the rabbits. For the period from 1½ to 5 months following operation the animals were, to outward appearances, normal in every way, they were lively, and plump, and had developed good appetites, but they showed complete lack of sexual interest. These experiments were performed in the early months of the year, December to May, when under normal conditions the does are coming on to heat after the comparatively quiescent period of winter. The two operated bucks, though, also showed this lack of desire, so that it is unlikely that the time of year was the decisive factor.

At the end of this period all the animals commenced to waste. In spite of every possible attention, anorexia developed, the animals becoming more and more emaciated until death occurred from 2 to 7 months after the operation.

On post mortem, all the animals showed a complete lack of visible fat. The sexual organs were extremely atrophied. The hypothalamus, united to the pituitary gland and the sella turcica, was taken in each case, and serially sectioned. After staining the sections with haematoxylin and eosin, the lesions were verified histologically (figs 4 and 5, Plate 15). In all the animals, it was found that distal to (that is, on the pituitary side of) the lesion the stalk and posterior lobe were shrunken and very cellular (compare figs 6 and 7 Plate 15). Also in two of the glands a large accumulation of colloid was observed in the remains of the posterior lobe (see fig 4). Gersh and Tarr (1935) have lately questioned the function of this colloid. They suggest it might be a histological artifact. If this is true, it is difficult to see why the colloid should increase in amount after lesions of the stalk. In the immediate vicinity of the lesion there was also a varying accumulation of small darkly staining cells, again sharply localized to the distal segment of the stalk. Crowe, Cushing and Homans (1910), and Cushing and Goetsch (1910) also noted these effects after similar experiments. The number of cells in the anterior lobe appeared greatly increased. The individual cells in this lobe, seemed to have lost most of their cytoplasm and this, together with the fact that the nuclei stained darkly with haematoxylin, gave the general appearance of a collection of small lymphocytes (figs 8 and 9 Plate 15). It was noticed that the anterior lobe was still very vascular.

According to Popa and Fielding (1930) there is no reason why lesions of the stalk should interfere with the systemic circulation of the pituitary gland, the arterial supply coming directly off the internal carotid artery, and a proportion of the venous drainage returning to the cavernous sinus. In this case, it follows that the atrophy of the pituitary noted above is probably due to interference with the nerve supply. It appears therefore, that lesions of the stalk lead to degeneration of the pituitary gland accompanied by the disappearance of sexual desire. As regards the cause of the degeneration, the results to be given seem in no way adverse to the suggestion just made, so that the degeneration observed may be due to lack of (perhaps, tonic secreto motor) nervous impulses rather than or in addition to, lack of blood supply.

#### STIMULATION OF THE PITUITARY GLAND AND HYPOTHALAMUS

In developing the technique for these experiments, I owe a deep debt of gratitude to Dr J Beattie and Professor W R Hess, both for their invaluable advice and for their kindness in permitting me to work for a short time in their laboratories in order to study their techniques.



The rabbits used were fully matured does that had been separated for at least 15 days, and in most cases for 30 days

As an anaesthetic, ether administered intratracheally was found to be most satisfactory. The trachea was easily sutured at the end of the experiment with two stitches, the only difficulty being that of cleaning the pharynx from mucous before suturing.

The method of stimulation was to insert the electrode through a small trephine hole, in the top of the skull, at bregma. It was always inserted

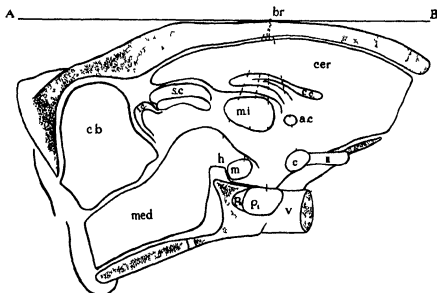


FIG 1.—Diagram of a sagittal section through the cranium of a rabbit, to show the paths taken by the electrodes ( $\times 2$  normal) A—B, anterior posterior tangent to the skull, through bregma, ac, anterior commissure, br, bregma, c, optic chiasma, cb, cerebellum, cc, corpus callosum, cer, cerebral hemisphere, h, posterior region of hypothalamus, ic, inferior colliculus, m, mammillary body, med, medulla, mi, massae intermedia, p<sub>1</sub>, anterior lobe of pituitary, p<sub>2</sub>, posterior lobe of pituitary, sc, superior colliculus, II, optic nerve, V, trigeminal nerve

in the mid line, but by inclining the head at varying degrees anterior posteriorly, different parts of the floor of the third ventricle could be stimulated

The apparatus designed for this purpose consisted of two parts (see figs 10 and 11, Plate 16), first a part for firm fixation of the head and for rotating the head into any desired position. The three points at which the head was clamped were the two posterior ends of the zygomatic arches and the upper incisor teeth. The second part was for insertion of the electrode

into the brain. The electrode was carried on a vertical rack, which could slide on a pivoted arm. Thus the electrode could be moved in any plane.

To reach the various parts of the hypothalamus, the following procedure was adopted. Sagittal sections were made through the heads of a number of rabbits, which had been fixed in 10% formalin for several weeks. These were photographed, enlarged prints were made and the various measurements taken. For each point of the hypothalamus stimulated it was necessary to know two facts, first the distance of that point from bregma, and secondly the angle contained between the line joining this point of the hypothalamus to bregma and a tangent of the skull drawn through bregma. (See fig 1.)

The average measurements obtained are given in Table I

TABLE I

	Distance of points from bregma cm	Angle between tangent to skull at bregma and line joining the point to be stimulated to bregma
Optic chiasma	1.60	75°
Tuber cinereum	1.65	90°
Mammillary body	1.80	99°
Posterior hypothalamus	1.65	110°
Pituitary gland anterior lobe	2.10	90°

The procedure for stimulating, for example, the posterior region of the hypothalamus, was as follows

Intratracheal cannula inserted. Rabbit's head fixed in the clamp. An incision  $2\frac{1}{2}$  in. long made in the skin at the top of the head. Head levelled transversely by means of a small oil level, placed on the two supra-orbital ridges and the two adjusting screws. The anterior-posterior tangent through the skull at bregma levelled by the same means. Head rotated through the required 20° to bring the posterior hypothalamus perpendicularly beneath bregma. A small hole, 1 mm in diameter, trephined through bregma, dura mater punctured. Electrode arranged with the tip directly over bregma and then racked perpendicularly down through 1.65 cm.

Various types of electrodes were used in the preliminary experiments. In the final experiments, however, unipolar stimulation was found easier to work with, since the finer electrodes could be inserted with less damage to the brain and also gave more constant results. The large diffuse electrode was a smooth brass rod inserted in the rectum, whilst the stimulating electrode consisted essentially of a fine steel wire, mounted in the holder of a hypodermic syringe needle and insulated with glass capillary tubing to

within 1 mm from the tip. The diameter of the wire was 0.26 mm and the external diameter of the glass capillary tubing 0.37 mm. The stimulating electrode was always made the cathode. The current used for stimulating was a direct, damped, pulsating current. The final circuit used is shown in

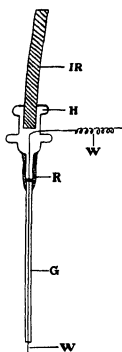


FIG 2

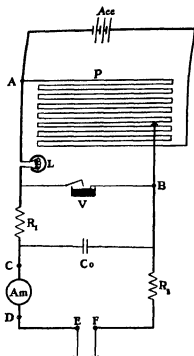


FIG 3

FIG 2—Diagram of electrode *G* glass capillary tubing, *H*, holder of hypodermic syringe needle, *IR*, rod of insulating material for attachment of electrode to clamp, *R*, insulating solution of rubber, *W*, steel wire

FIG 3—Diagram of the final circuit used *A B*, voltages tapped off potentiometer, measured across these two points, *Acc*, three 2 V accumulators, *Am*, milli ammeter, *C-D*, current passing through the animal measured across these two points, *Co*, condenser of 4  $\mu$ F capacity, *E-F* potential difference across the electrodes, measured from these two points *L*, small lamp of 4.5 V, *P*, potentiometer, *R<sub>1</sub>*, *R<sub>2</sub>*, resistances of 1000 ohms *V*, contact breaker, 5-8 c/sec

fig 3 Three 2.0 V accumulators were placed in series with a potentiometer, consisting of 20 m of 20 s w g eureka wire, from which a voltage varying between 3.0 and 4.0 V was tapped off. The potential difference measured across the electrodes is, of course, not the same as that tapped off the potentiometer. Voltages of 3.0, 3.5, 3.75 and 4.0 V, as tapped off the

potentiometer, from *A* to *B*, are equivalent to potential differences of approximately 1.5, 1.7, 1.8 and 1.9 V across the electrodes from *E* to *F*. The currents varied between 0.4 and 0.9 mA. The contact breaker was arranged to vibrate at 5-8 c./sec. The two resistances  $R_1$  and  $R_2$  were each of 1000 ohms, and the capacity of the condenser was 4  $\mu$ F. A circuit very similar to this and its properties has been fully described by Hess (1932).

In the preliminary experiments an induction coil was used. This has one grave disadvantage. The autonomic fibres of the hypothalamus, or at least those subserving ovulation, appear to have, to undamped currents, a very high threshold of stimulation relative to the threshold of the somatic fibres in the pyramidal tracts that lie in close postero-lateral relationship to the hypothalamus. Thus it follows that to stimulate the autonomic fibres comparatively high voltages must be used, which in turn stimulate the pyramidal tracts causing the muscles of the head, neck and fore limb to twitch so that it is impossible to keep the head perfectly still. This causes the head to move relatively to the inserted electrodes and damages the brain. When damped currents are used, the threshold for the somatic fibres, though still lower than that for the autonomic fibres, is relatively much closer. Thus strong currents may be used without the disadvantageous spread of stimulation to the pyramidal tracts.

Except for the preliminary experiments, the time periods of stimulation were half a minute "on" and half a minute "off", repeated ten times, then five minutes "off". This was repeated four times, giving a total period of stimulation of 20 min. spread over 1 hr. These time periods were kept the same for all the later experiments so that a comparison could be drawn between the effects obtained from stimulation of different points.

The only symptoms noted during stimulation were a flickering of the eyelids, nictitating membranes and eyeballs. Sometimes this occurred in one eye, sometimes in both, depending on the accuracy with which the electrode was inserted in the mid-line. It is fairly certain that this effect is due to direct spread of current to the third nerve, and excitation of the somatic fibres therein. No changes in the size of the pupils were observed, so presumably the autonomic fibres in the third nerve were not stimulated. These effects were the same, whether the electrode was in the region of the tuber cinereum or passed through the hypothalamus into the pituitary gland.

At the end of stimulation, the head wound and trachea were sutured. In no case were any aseptic precautions taken, for the animals were killed before septic complications could occur. In nearly every case, uneventful and quick recoveries occurred.

The animals were killed 30-120 hr. following stimulation.

In the earlier experiments, in the cases of negative results, it was found to be difficult to say whether the animals were on heat or not. And as ovulation never occurred in an anoestrous or pregnant rabbit, the difficulty thus arose as to whether the negative effect was due to the rabbit not being on heat, or due to defective stimulation. Sections were made through the uteri of these rabbits but in several cases these were found to give insufficient evidence to decide this point. In the later experiments, the stimulated does were put with a buck immediately prior to killing and the act of copulation taken as an indication that they were on heat at the time of stimulation. The results quoted later include only those rabbits which definitely were in oestrous at killing.

The histological work consisted of sectioning the uteri in cases of negative results and the ruptured follicles in cases of positive or questionable results. 10% formalin was used as the fixative and haematoxylin with eosin as the stains.

The heads of the animals were injected with 10% formalin, and then fixed in this solution for 2-3 weeks. Careful dissection of the hardened brain then showed the point of stimulation fairly accurately, for the electrode usually left a fine trail of blood, whilst in hypothalamic stimulation around the point of stimulation was either a yellow discoloration or a slight haemorrhage. The cause of the latter is unknown. In pituitary stimulation, the position of the electrode was gauged by the position of the haemorrhage in the gland. The extent of this varied considerably, but bore no relationship to the results of the stimulations.

#### RESULTS OF DIRECT STIMULATION OF THE PITUITARY GLAND

The pituitary gland was reached by inserting the stimulating electrode at right angles to the tangent of the skull at bregma and racking it down 2.0-2.1 cm, according to the size of the rabbit.

*In the first experiment*—the stimulating electrode was a fine copper wire, s.w.g. 32, lacquered to the tip. Only the transverse section at the end was bare. This was inserted 2.0 cm through bregma. The non-stimulating electrode was a lead knob inserted through a small incision in the soft palate to lie under the basisphenoid. Stimulation was by means of an induction coil, the secondary coil being placed as near to the primary as was possibly consistent with low spread of stimulation. The symptoms on stimulating were slight stiffening of the neck muscles, flickering of the eyelids, eyeballs and nictitating membranes and pupil constriction. The first of these is due to spread to the pyramidal tracts and the latter to spread

to the third nerve. Stimulation lasted for 6 min., distributed at intervals over 1 hr. This animal was killed 51 hr following stimulation, and on post mortem 9 ovulated follicles were discovered in the ovaries

*In the second experiment*—similar electrodes were used, but the circuit, as shown in fig 3, had a maximum potential difference of 3.0 V between *A* and *B*. No symptoms were observed during stimulation. Thirty hours later the animal was killed and the ovaries found to contain 6 ovulated follicles and 3 haemorrhagic follicles

*In the third preliminary experiment*—the anode was again the lead knob inserted through the soft palate, but the stimulating cathode was a steel wire insulated with glass capillary tubing (fig 2). The tip was bared for only  $\frac{1}{2}$  mm instead of the usual 1 mm. Again the direct, damped form of current was used, this time with a potential difference of 4.0 V between *A* and *B*. The animal recovered quite normally but was exceptional in that it was found dead 44 hr. post-stimulation. In the ovaries were 6 ovulated follicles and 5 abnormally large follicles which it is believed were becoming cystic

After these three experiments it was decided to use the same electrode types and stimulating currents as were being used in the concurrent experiments on hypothalamic stimulation, thus making it possible to compare the current strengths necessary to give positive results from pituitary and hypothalamic stimulation. As described previously, these electrodes consisted of a stimulating cathode of fine steel wire, insulated to within 1 mm of the tip by glass capillary tubing, with a large diffuse anode placed in the rectum. The current was of the direct, damped, pulsating type (fig 3).

For the results of these experiments see Table II. It will be seen that stimulation with a potential difference of 3.5 V between *A* and *B* was applied to eight rabbits. Of these, seven were stimulated for the usual time of 1 hr. and showed no ovarian effects. The eighth rabbit was stimulated in exactly the same manner, but the stimulation lasted half an hour longer. Although no ovulation occurred, some secretion of pituitary hormones presumably must have taken place, for one cystic follicle and two large haemorrhagic follicles were found 75 hr after stimulation. For the seven rabbits stimulated at 3.5 V, with the "standard" time intervals and by the "standard" method, 100% negative results were obtained.

Three rabbits stimulated with 3.75–3.8 V across *A–B* gave two positive results. In one case ovulation and in the other case haemorrhagic and cystic follicles were obtained.

Stimulation with 4.0 V across *A–B* was applied to eleven rabbits giving six positive and five negative results. That is a response of 54.5%.

TABLE II

No. of rabbit	Past history	Strength of stimulating voltage	Hours between stimulation and killing	Ovaries on post mortem
		A-B V		
49	Parturition 12 days previously	3.5	73	8 moderately ripe follicles, 8 very ripe follicles
50	Parturition 18 days previously	3.5	74	10 moderately ripe follicles; 7 ripe follicles
62*	Separated 19 days	3.5 Stimulated for 1½ hr	75	1 cystic follicle, 2 haemorrhagic follicles
63	Separated 20 days	3.5	60	2-3 old corpora lutea in each ovary, 2-3 follicles in each ovary, slightly larger than normal
71	Parturition 14 days previously	3.5	65	10 ripe follicles
72	Separated 15 days	3.5	52	7 ripe follicles
74	Parturition 8 days previously	3.5	Laparo- tomy at 51	Several follicles red and ripe in each ovary
75	Separated 16 days	3.5	45	9 ripe follicles
52*	Separated 21 days	3.8	74	7 ovulated follicles, 10 large follicles
60*	Separated 32 days	3.75	51	5 follicles in various stages of becoming cystic and haemorrhagic
61	Separated 34 days	3.75	51	14 ripe follicles
53	Parturition 21 days previously	4.0	73	2-3 ripe follicles in each ovary
55*	Separated 41 days	4.0	51	7 ovulated follicles, 1 follicle ovulated partly haemorrhagic
58*	Parturition 23 days previously	4.0	75	4 ovulated follicles, 1 ovulated follicle partly haemorrhagic, 4 cystic follicles, 1 haemorrhagic follicle
59*	Separated 30 days	4.0	51	10 ovulated follicles
64*	Separated 30 days	4.0	52	8 ovulated follicles, 2 large haemorrhagic follicles, 7 small haemorrhagic follicles
66*	Separated 25 days	4.0	51	2 ovulated follicles, 4 large haemorrhagic follicles, 1 very swollen follicle, partly haemorrhagic
68	Parturition 11 days previously	4.0	53	8 ripe follicles
69	Parturition 14 days previously	4.0	50	9 ripe follicles
76	Separated 22 days	4.0	42	9 ripe follicles
77	Separated 40 days	4.0	80	11 ripe follicles
78*	Separated 55 days	4.0	78	5 ovulated follicles, 3 haemorrhagic follicles

\* Indicates a positive result

In the three preliminary experiments the results were also positive.

In all, effects on the ovaries of eleven rabbits have been shown after passage of electric currents of various forms through the pituitary glands. It is felt, from scanty evidence, that the most uniform effects would be obtained with an anode in the form used in the preliminary experiments inserted through the soft palate so as to lie beneath the basisphenoid bone. This condenses the current flow through the pituitary gland. In the present experiments it was necessary to have electrodes similar to those used for stimulating the hypothalamus so that the comparison could be made between the relative strengths of current needed to elicit responses in the two regions.

It might be suggested that the presence of the needle electrode in the gland and the damage performed were sufficient to liberate enough hormone into the blood stream to cause ovulation. That this is not so is shown by the fact that stimulation with the voltage of 3.5 V gave 100% negative responses, the damage caused to the gland being as extensive in some of these experiments as in those in which positive responses were obtained by stimulation with 4.0 V.

Again, it might be suggested that the reason for negative responses is that during stimulation the insertion of the electrode through the floor of the third ventricle damaged some structure essential to the mechanism of ovulation. To settle this point, a rabbit was stimulated in the usual manner with a voltage of 3.5 V. Fifty hours after stimulation this doe rabbit was allowed to copulate with a buck. Immediately after copulation, a laparotomy was performed and both ovaries carefully examined. There was no sign of ovulation or haemorrhagic follicles. The animal was killed 70 hr post stimulation, when five ovulated follicles were found in the ovaries. This demonstrates the fact that even after completion of the experiment, when the hypothalamus has been pierced and when the pituitary gland has been damaged, the animal can still ovulate in a perfectly normal manner following copulation.

The time of ovulation in these pituitary stimulations was placed roughly between 15 and 40 hr after stimulation. This was estimated from a study of serial sections through the ovulated follicles.

#### RESULTS OF STIMULATION OF THE HYPOTHALAMUS

In the earlier experiments, stimulation of the hypothalamus was attempted by use of the induction coil. The electrodes used were of the concentric type. The results obtained were uniformly negative for the reason given above, that is, owing to the spread of the faradic current to



the somatic nerve fibres it was impossible to apply a strong enough current. They show, however, that the mere presence of the electrodes in the hypothalamus, though thicker than those eventually used, is insufficient to cause ovulation.

Later, direct pulsating damped currents were used for stimulating, obtained from the circuit already described, or similar circuits.

In the following account results obtained from the latter circuits have been classed together, and are alone considered.

#### *1—Stimulation of the Region of the Tuber Cinereum*

The region of the tuber cinereum was first stimulated on the supposition that if nerve fibres which influence the anterior lobe passed via the stalk, they would converge at the tuber. The region of the tuber is taken to mean that part of the hypothalamus lying inferior to a line joining the optic chiasma to the mammillary body. This region can be reached by racking the electrode down 1.65 cm. below bregma at an angle of  $90^\circ$  to the tangent to the skull at this point.

In the first five experiments the concentric needle electrodes were used, the central electrode being made the cathode. The potential difference from *A* to *B* was 3.5 V. Four out of these five rabbits ovulated, in three cases haemorrhagic follicles were also formed.

In the other fifteen cases, unipolar stimulation was used. The electrodes were here similar in every detail to those used in the later experiments upon the stimulation of the pituitary, that is, the stimulating cathode was as shown in fig. 2, with a large anode in the rectum.

Two rabbits were stimulated with a potential difference of 3.0 V from *A* to *B*. One of these killed 100 hr. later showed many large cystic and haemorrhagic follicles in the ovaries (see fig. 13, Plate 17). The other gave a negative result.

Two rabbits were stimulated with a potential difference of 3.5 V from *A* to *B*. One of these killed 50 hr. later showed nine ovulated follicles and one cystic follicle turning haemorrhagic in the ovaries. The other gave a negative result.

Eleven rabbits were stimulated with a potential difference of 4.0 V from *A* to *B*. On killing, six of these rabbits showed ovulated follicles together with various forms of cystic and haemorrhagic follicles. The other five gave negative results.

In all, twenty rabbits were stimulated around the region of the tuber cinereum, twelve giving positive results.

*2—Stimulation of the Tuber Cinereum after Cervical Sympathectomy*

In three rabbits the superior cervical ganglia and upper half of the cervical sympathetic chain were removed on both sides. Six weeks later, the tuber cinereum was stimulated, with a potential difference of 4.0 V from *A* to *B*. One of these three rabbits gave a positive response, for, on killing 72 hr. later, nine ovulated follicles and one haemorrhagic follicle were discovered in the ovaries. The other two rabbits gave negative results.

*3—Stimulation of the Posterior Hypothalamus*

The exact position of the region stimulated is shown in fig. 15, Plate 17, a region not very far removed from the red nucleus and its connexions. This proximity led to complications.

Out of four rabbits stimulated in this region, three on recovery exhibited signs that the mechanism of head posture had been disturbed. In the normal standing position, the heads were rotated with the left eyes upwards. On holding the rabbits in mid-air, the left fore-limbs and hind-limbs extended, whilst the limbs of the right side flexed. It is interesting to note that the damage was mainly to the left of the mid-line in all three cases, so that the rigidity was ipsilateral. The reason for this is not clear.

These three animals showed no ovarian effects following stimulation.

The fourth animal made a very good recovery with none of the above effects. On killing 100 hr. after stimulation the ovaries were found to contain ten ovulated follicles and nine small haemorrhagic follicles. Dissection of this animal's head is shown in fig. 15, Plate 17.

Thus, out of the four animals stimulated in this region, the one which recovered to normality was the one which ovulated. It is quite possible that the damage prevented the other three showing the ovarian reaction.

*4—Stimulation of the Hypothalamus in a Ferret*

Dr F. H. A. Marshall (private communication) stimulated ferrets through the heads with the large electrical shocks that were successful in inducing ovulation in rabbits and pseudo-pregnancy in rats. With ferrets, no reaction on the ovaries could be obtained by this method. This may be due to one of two reasons. First, that ferrets normally take a very long time to copulate, any period up to 1 hr. or over. If the male is taken from the female after a quarter of an hour, ovulation may not follow. Thus to reproduce the natural conditions, it is possible that stimulation would have

to be applied for an equal time. Secondly, the electrical resistance of the bones of a ferret's skull may be greater than that of rabbits and rats, in which case, not so much current would pass through the hypothalamus and pituitary gland, for, in this method of stimulation, the electrodes were external to the cranium. With these two points in mind, it was thought to be of interest to see whether direct stimulation of the hypothalamus by the present technique would affect the ovaries, for the stimulation point would be inside the skull and the intermittent stimulation would last for 1 hr.

The electrode was inserted into the posterior region of the hypothalamus, 2 mm above the superior surface of the pituitary gland. The potential difference between *A* and *B* was 4.0 V and stimulation was applied for the usual time intervals over 1 hr.

On recovery, this animal was discovered to have disturbed head posture similar to some of the rabbits stimulated in this region. Unlike rabbits, however, at post mortem, 102 hr after stimulation, three cystic follicles which had undergone quite extensive lutealization were discovered in the ovaries.

Owing to the fact that the animals were not killed until 2-3 days after stimulation, it was found difficult to place accurately the time of ovulation. From a study of sections, it appears that ovulation may occur at any time between 10 and 60 hr following stimulation.

#### DISCUSSION

Previous workers have noted various effects on stimulation of the pituitary gland. Cyon (1898, 1899, 1900) obtained slowing of the heart with increased amplitude of the beat on stimulation by pressure or electrical excitation. Weed, Cushing and Jacobson (1913) showed stimulation of the exposed pituitary in dogs gave variation in the blood sugar. This they put down to stimulation of the posterior lobe. Keeton and Becht (1915) also found that stimulation in dogs produced glycosuria, but not if the splanchnic nerves had been previously cut. It is possible, therefore, that this effect is not due to action of the pituitary gland.

The experiments described above have shown that ovulation in the rabbit can be obtained by electrical stimulation of the pituitary and the hypothalamus. There is evidence that the effects originate definitely in the gland, or through the intermediation of the gland, for the effects upon the ovary are very similar to those obtained by the injection of extracts, either of the pituitary or of pregnancy urine. These effects, besides those

of normal ovulation, include the production of cystic follicles,\* of cystic follicles becoming haemorrhagic and of large haemorrhagic follicles (figs 12, 13, 14, Plate 17)

One difference between stimulation and injection of extracts is that the injection of extracts will produce ovulation, formation of luteal tissue, and haemorrhagic follicles in the immature and anoestrous rabbit, but so far as has been observed, to obtain any result by electrical excitation the rabbit must be well on heat. It is possible that after a more delicate technique for stimulating this gland has been obtained, prolonged stimulation over many days might produce different results.

As regards the conclusions to be drawn from the results of the hypothalamic stimulation, one difficulty arises. It is just possible that the positive results obtained were due to general spread of the stimulating current to the pituitary gland, and not due to stimulation of nerve fibres running through the hypothalamus.

At first sight the obvious experiment to perform is stimulation of this region after preliminary transection of the stalk. This is technically extremely difficult, for, as shown previously, after section of this structure, the animals enter into a state of anoestrous. Therefore, unless the stimulation was carried out immediately following the preliminary operation, the experiment would be useless. If the two operations were performed together, the time of the whole experiment and the damage committed would be so great that almost certainly no results of value would be obtained.

Another possible way of deciding the question would be to try to define more accurately the exact regions in the hypothalamus from which the reactions could be obtained and to follow the probable nervous pathway through the mid-brain, so increasing the distance between the stimulating electrode and the pituitary gland. This would require a more delicate stereotaxic instrument than was available in the present research, and further that rabbits of the same breed and nearly uniform in size should be used, thus ensuring more accurate localization when inserting the electrode.

Concerning this question, the evidence at present available is

(1) That during hypothalamic stimulation, no changes in pupil size were observed, although the eyeballs, eyelids and nictitating membrane were

\* One observation may be of agricultural interest. In one animal (rabbit 60), cystic and haemorrhagic follicles were obtained following pituitary stimulation (fig 12, Plate 17). Fifty hours after stimulation and just before being killed, this rabbit was put with a buck. The experimental animal showed intense sexual excitement, manifested by continuous "jumping" on to the male rabbit. The condition produced by this stimulation is thus very suggestive of the pathological condition, nymphomania.

seen to flicker. The conclusion drawn is that the spread of current to the oculomotor nerves was sufficient to stimulate the somatic fibres, but insufficient to stimulate the autonomic fibres which have a higher threshold of excitability. Now in the rabbit oculomotor nerves he in closer anatomical relationship to the tuber cinereum than does the pituitary gland, so that it would be justifiable to assume that if the current spread did not excite the autonomic fibres in the third nerve it would not excite fibres of a presumably similar nature in the gland.

(2) The results show that the threshold current needed to produce ovulation was the same whether the electrode was in the hypothalamus or in the pituitary. This would indicate that spread of current during hypothalamic stimulation would be insufficient to account for the results obtained.

(3) In rabbit 17, stimulated in the posterior hypothalamus (see fig. 15, Plate 17), the tip of the electrode was 4-5 mm. distant from the nearest point of the pituitary gland. This animal ovulated about 35 hr. post-stimulation. After taking into consideration the fact that, in a case of intended pituitary gland stimulation with the same voltage in which the electrode was found on post-mortem to be lying outside the gland but directly adjacent to it, no ovulation was obtained, it seems safe to conclude again that the results were not due to spread of current.

Therefore, the evidence at present indicates strongly, though not certainly, that ovulation in the rabbit may be induced by stimulation of nerve fibres in the hypothalamus as well as by pituitary stimulation.

If this view be accepted, it would follow that the hypothalamus forms part of a reflex path used in stimulating the pituitary gland after sexual excitement. It therefore becomes of considerable interest to discuss the pathway in detail.

It seems highly probable that the stimulation of the pituitary directly is acting on non-medullated nerve fibres in the anterior lobe and that these fibres have a very high threshold of excitability. The functional links between the hypothalamus and the anterior lobe of the pituitary that have been suggested are, the cervical sympathetic nerves, the greater superficial petrosal nerves and the pituitary stalk. Of these, it cannot be the cervical sympathetic nerves alone, for, as previously stated, ovulation can be obtained in a normal fashion after extirpation of these nerves. It may be the greater superficial petrosal nerves, but on this point there is little evidence. It appears more probable that the functional link is the pituitary stalk. The main evidence for this is that genital atrophy follows lesions of the tuber cinereum and pituitary stalk which has been noted

by several previous workers and confirmed in the rabbit in this present account.

The hypothesis that the hypothalamus influences the anterior lobe via nervous impulses passing down the stalk is open to one obvious objection that is, the fact that nerve fibres have never been seen passing from the posterior to the anterior lobe. By the use of the de Castro technique of staining, nerve fibres may be seen passing from the pars nervosa to the pars intermedia (Cushing 1932*b*). Serial sections through several rabbit pituitaries have been obtained, demonstrating this fact. It is difficult to trace these fibres to their termination owing to their extremely fine character. It may be mentioned in passing that although the pars nervosa and intermedia may take the stain perfectly, the pars anterior takes it poorly. In order to surmount the above objection, it is necessary to suppose either that the nerve fibres seen to enter the pars intermedia eventually pass round the cleft into the anterior lobe, or else that the posterior or intermediate lobe can influence the anterior lobe hormonally. The former supposition is felt to be the more probable of the two.

This suggested pathway (hypothalamus, stalk, posterior lobe, anterior lobe) may possibly be active in several phenomena which have previously been difficult to explain. It is well known that in some birds and in the ferret, the sex cycle may be influenced by extra radiation. It is probable that the radiation, at least in the ferret, acts on the anterior lobe of the pituitary through the intermediation of the eyes (Bissonnette 1936). Collin (1935) has brought forward evidence that there is a nervous connexion passing from the optic tract through the hypothalamus to the stalk of the pituitary. On correlation, these two facts fit in well with the above theory.

Again, it is highly probable that the uterus may affect the anterior lobe of the pituitary gland by nervous reflex paths, for it has been shown that hysterectomy in the pseudo-pregnant rabbit, or guinea-pig, will prolong the life of corpora lutea in the ovaries (Loeb 1923, 1927, Loeb and Smith 1936, Asdell and Hammond 1933). Selye (1934) showed in rats that Caesarian section initiates lactation and the recurrence of oestrous cycles, though this does not occur if the uterus is distended with wax after removal of the foeti.

From the work of Selye and McKeown (1934), it appears that mechanical stimulation of the nipples is the cause of lactation dioestrus in rats and mice.

Thus there is evidence that the anterior lobe may be influenced by nervous effects from the eyes, uterus and mammary glands, as well as from the vaginal region. Also, Haterius (1933) produced evidence that a psycho

factor normally plays a part in the induction of pseudo-pregnancy in rats, whilst Theobald (1936), who has collected clinical evidence for a diencephalic centre governing the menstrual cycle in women, states that psychic factors such as fear of pregnancy and hypnosis may affect this cycle through the supposed centre

It might be theorized that the hypothalamus controls the secretion of hormones, other than the gonadotropic hormone, from the anterior lobe. In a recent review on the relationship between the hypothalamus and the pituitary, Dodds and Noble (1936) draw attention to the well-known facts that hypothalamic damage may cause glycosuria, adiposity, and genital atrophy, which are possibly all anterior lobe effects. Here then is evidence that the hypothalamus controls the secretion of the hormones influencing sugar and fat metabolism. There is no reason to doubt that the thyrotropic, adrenotropic, lactogenic, parathyrotropic and growth hormones are not similarly controlled.

Finally, much evidence has accumulated in the past few years indicating that the hypothalamus contains centres controlling the autonomic nervous system. If it is further shown that the pituitary gland and, through the intermediation of the structure, the thyroid, parathyroids, adrenals and gonads are likewise influenced by this important region of the brain, then it would be possible to say that a very large part of both the nervous and chemical links which unite one part of the body functionally with another part are controlled by this region of the diencephalon.

I wish to express my sincerest thanks to Professor H. A. Harris and Professor E. D. Adrian for their ever willing advice. In particular, my deepest gratitude is due to Dr F. H. A. Marshall, first, for suggesting this work to me, and secondly for his constant aid and encouragement. Also I should like to thank Mr E. Powell for the care he bestowed on the animals.

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#### SUMMARY

Lesions in the stalk of the pituitary gland have been shown to cause genital atrophy in male and female rabbits.

Direct stimulation of the pituitary gland in female rabbits induced ovulation 15-40 hr later and sometimes the formation of cystic and haemorrhagic follicles.

Stimulation of the hypothalamus gave results similar to the above.



FIG. 4



FIG. 5

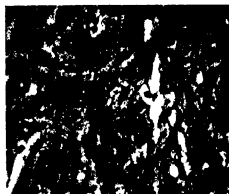


FIG. 6



FIG. 7

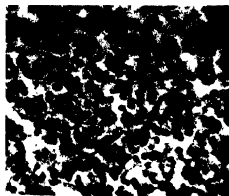


FIG. 8



FIG. 9



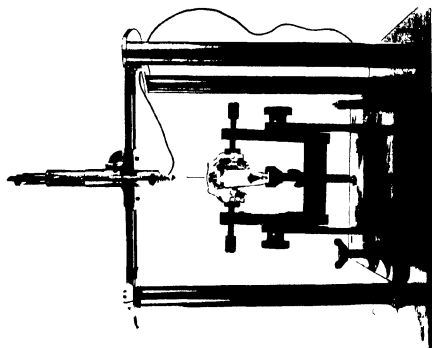


FIG 11

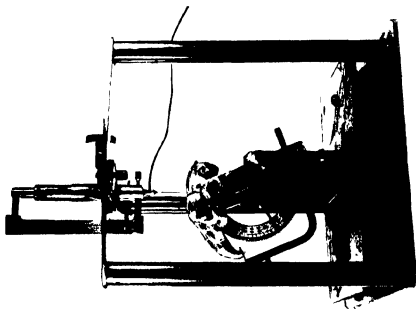
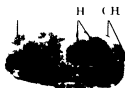


FIG 10



11 11



13 13



14 14



15 15



Evidence is adduced that this is an effect on nerve fibres in the hypothalamus rather than due to spread to the pituitary gland

The control of the anterior lobe of the pituitary by the hypothalamus is discussed, with particular reference to the nervous pathway involved

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## DESCRIPTION OF PLATES

## Plate 15

- FIG 4—Sagittal section through pituitary gland, stalk and hypothalamus. Note atrophic appearance of pituitary gland, line of lesion (indicated by two arrows), vacuoles in posterior lobe and stalk (previously filled with colloid) *p*, posterior lobe, *i*, intermediate lobe, *a*, anterior lobe ( $\times 26$ )
- FIG 5—Line of lesion (in fig 4) under higher power. Note fibrous tissue "healing", and slight accumulation of basophilic cells *B* ( $\times 72$ )
- FIG 6—Normal posterior lobe of pituitary gland ( $\times 360$ )
- FIG 7—Posterior lobe of experimental animal, after stalk lesion. Note the great increase in basophilic cells and the cavities previously filled with colloid ( $\times 360$ )
- FIG 8—Normal anterior lobe of pituitary gland ( $\times 360$ )
- FIG 9—Anterior lobe of experimental animal after stalk lesion. Note reduction of the cell cytoplasm, and the apparent increase in the number of the cells ( $\times 360$ )

## Plate 16

- FIG 10—Side view of stimulating apparatus. Note that the apparatus consists of two parts—one for clamping and rotating the head, and the other for inserting the electrode ( $\times \frac{1}{2}$ )
- FIG 11—Front view of same apparatus ( $\times \frac{1}{2}$ )

## Plate 17

- FIG 12—Ovary from rabbit 60, killed 50 hr after stimulation of pituitary gland. Two of the follicles became very protuberant and showed some internal haemorrhage, though unruptured externally. A third follicle became haemorrhagic ( $\times \frac{3}{2}$ )
- FIG 13—Ovary from rabbit 24, killed 100 hr after stimulation of the tuber. This ovary showed one large, clear cystic follicle, *c*, two cystic follicles becoming haemorrhagic, *ch*, and two large haemorrhagic follicles, *H* ( $\times \frac{3}{2}$ )
- FIG 14—Ovary from rabbit 38, killed 50 hr after stimulation of the tuber. This ovary showed one large, clear cystic follicle, *c*, four cystic follicles becoming haemorrhagic, *ch*, and one young corpus luteum ( $\times \frac{3}{2}$ )
- FIG 15—Head of rabbit 17, sagittal section. This rabbit was stimulated in the posterior hypothalamus, and killed 100 hr later (The ovaries showed ten young corpora lutea and nine small haemorrhagic follicles). The dissection shows the point of stimulation quite clearly. The area surrounding the electrode showed necrosis with a pale pink blood clot (The pink colour of the clot is greatly intensified in the photograph, appearing black in reproduction) ( $\times 2$ )

Studies on the Nature of the Amphibian  
Organization Centre  
V—The Distribution and Nature of Glycogen in  
the Amphibian Embryo

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(*Communicated by Sir F. Gowland Hopkins, O.M., F.R.S.*—

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# 1—INTRODUCTION

In an earlier paper (Heatley 1935) an account was given of a method of estimating glycogen in amounts of tissue of the order of 1 mg., and some preliminary results were described of an investigation into the distribution of glycogen in the different regions of the amphibian gastrula. This work has been extended during the last winter, with more rigid experimental conditions. The blastula as well as the gastrula has been studied, and the regions selected for analysis have been more clearly defined. An attempt has also been made to estimate the relative amounts of lyo- and desmo-glycogen in each region. The dissections were done by both collaborators, and all the chemical work by one of us (N. G. H.).

# 2—METHODS

*a—Material*—Axolotl eggs which had been laid spontaneously were removed from the tanks and allowed to develop at room temperature, but on many occasions they were placed temporarily in the ice-chest to delay development. Embryos described in the following as being in the "blastula" stage had just perceptible blastopores, whilst those in the "gastrula" stage ranged from those with a not quite spherical blastopore to those with a medium-sized yolk plug. In terms of the standard tables prepared by Glaesner (1925), the blastula would include stages 8 and 9, and the gastrula stages 10 and 11.

*b—Selection of Regions*—In the previous paper (Heatley 1935) the regions analysed were merely 'dorsal ectoderm', 'ventral ectoderm', 'mesoderm' and 'endoderm'. In the work described in this paper a new method has been adopted in both stages two lateral segments were cut from the embryo and discarded, leaving a disk about equal in thickness to the radius of the original embryo. In the blastula this disk was divided by six radial cuts, each portion being named as shown in fig. 1. In the gastrula stage the tissue was taken from the position to which, according to Vogt (1929), it will have migrated during the invagination, thus it has been assumed that the whole of the mesoderm and all the dorsal ectoderm of the gastrula were originally D 1 and D 2 in the blastula. D 1 will in time become neural tube. D 2 the invaginated roof of the archenteron.

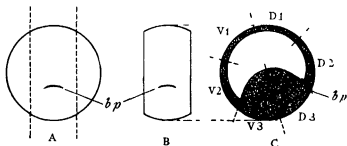


FIG. 1—See text. *bp* = position of blastopore.

and subsequently all the mesoderm, V 1 and V 2 all skin, and V 3 and D 3 all endoderm, hence viscera. No attempt was made to divide D 3 and V 3 in the gastrula, they were estimated together.

All dissection was done by means of finely drawn out glass needles.

*c—Collection of Tissue*—In the earlier work the tissue was plunged into absolute alcohol, from which it was subsequently removed, dried, and weighed. It was pointed out that different amounts of alcohol soluble material might thus be lost from the different regions, giving a false comparison of their glycogen content. In the present work the tissue has been collected in the following, different, way. A series of clean tubes (250 × 6 mm) were accurately weighed on the micro balance, and 0.2 ml of absolute alcohol was measured out into each. As the tissue was dissected from each embryo it was transferred by means of a teat pipette to a very small spatula with a cup shaped depression. Excess moisture was removed, and the spatula was plunged into the appropriate tube and agitated until the tissue became detached. When sufficient material had been collected,

the tube was dried in a stream of dry air at 100° C for 2 hr. It was shown that with the largest amount of tissue ever taken constant weight was attained in less than 1½ hr. and also that alcohol alone left no detectable residue. The tube was carefully wiped while still hot first with a damp and then with a dry chamois leather and finally reweighed.

*d—Chemical Estimation*—The chemical estimation was performed in exactly the same way as described in the original paper except that the 30 % potash in which the tissue had been dissolved was diluted with an equal volume of 0.5 % sodium sulphate solution instead of with distilled water.

Trouble was experienced with the actual sugar estimation until it was realized that a considerable excess of iodine is essential. In some of the estimations where there was expected to be more glycogen than usual two volumes of iodine were taken instead of one. Under these conditions however the relation of iodine absorbed to reducing sugar present was not linear but all those values given below for which two volumes of iodine have been taken have been corrected from appropriate curves. The large scatter in the figures for lyo glycogen is probably due to this cause since greater amounts of tissue were used in these experiments.

*e—Separation of Desmo and Lyo glycogen*—The estimation of the desmo and lyo fractions of glycogen separately in this tissue presented considerable experimental difficulty. It was no longer permissible to fix the material in alcohol as soon as it had been dissected and as the dissection of sufficient material for one experiment took a considerable time there was grave risk of glycolysis. This was reduced as much as possible by keeping the dissected tissue in ice. Willstatter and Rohdewald (1934) have described two ways of isolating lyo glycogen: one consists of extracting the tissue with ice cold trichloroacetic acid and the other of extracting it with boiling water. The latter procedure was chosen in this case since the use of trichloroacetic acid would have introduced serious complications in the determination of the amount of tissue present. The procedure finally adopted for the separation of these two fractions was as follows:

- (1) The tissue was collected in tared titrating tubes measuring 250 × 6 mm each containing 0.2 ml of distilled water and cooled in ice.
- (2) When sufficient material had been collected the tubes were suspended in a cage which was plunged into a current of steam. (The heating must have been rapid enough to prevent any appreciable glycolysis since 0.2 ml of alcohol under similar conditions boiled in less than 2 sec.)
- (3) The tubes were completely surrounded by steam (to prevent evaporation) for 10–15 min during which time they were shaken at



intervals (A mechanical shaker was made for this purpose from a loud speaker unit connected through suitable resistances to the A.C. lighting supply. During the shaking the tubes were still surrounded by steam.)

(4) The tubes were centrifuged.

(5) The clear centrifugate was quantitatively transferred to a second series of tared tubes by means of a special capillary syphon.

(6) The tubes containing the residue were heated and shaken with a further 0.2 ml. of distilled water whilst those containing the centrifugate had the volume of their contents reduced by evaporation.

(7) The tubes containing the tissue in suspension were centrifuged again and the centrifugate in each case was transferred to the same tube which had received the first centrifugate from that particular sample.

One or sometimes two more extractions were made in exactly the same way then both sets of tubes were dried in a current of dry air at 100° C. for 2 hr. and after wiping were reweighed. The sum of the increase in weight of the two tubes of each pair represents the dry weight of the tissue taken. 30% potash was added to each tube and the glycogen was estimated in the usual way.

Preliminary experiments had shown that under these conditions most of the lyo glycogen is removed by two extractions, a third extraction removing an insignificant further amount. In all the experiments quoted three extractions were made except in the case of the regions D 3 plus V 3 where four were performed since the combined bulk of these two samples is considerably greater than that of the others.

### 3—RESULTS

These have been summarized in Tables I and II in which glycogen has been expressed as percentage of the dry weight of the tissue.

Table I gives the percentage of total glycogen in the different regions of the blastula and the gastrula. The second class results (in italics) were obtained in experiments where the weight of the tissue could not be determined with the usual accuracy; they have not been included in the calculation of the means or in the statistical treatment. The latter consisted of the application of the *t* test of Fisher (1935) and the values of *P* which were obtained show that only in the region D 2 can the decrease in glycogen content be considered as significant. The values for D 3 and V 3 though determined separately in the blastula stage have been pooled for the statistical analysis. In the case of these regions the value of *P* may be considered to be on the borderline of significance.

Table II summarizes the results of four experiments where desmo and lyo glycogen were estimated separately. The *t* test has been applied here to the sum of these two fractions in the same way that it was applied in Table I but the scatter is so large that the decrease of total glycogen in D 2, although considerable is no longer statistically significant (there is reason to believe that the scatter is mainly confined to the lyo glycogen figures, since as mentioned above special errors may arise in the estimation of the large amounts of reducing sugar in these fractions).

TABLE I—TOTAL GLYCOGEN IN AXIOLOTL EMBRYO SECOND CLASS  
RESULTS (IN ITALICS) HAVE NOT BEEN INCLUDED IN THE  
CALCULATION OF THE MEAN OR OF *P*

Exp	Dorsal 1		Dorsal 2		Ventral 1		Ventral 2		Dorsal 3 + Ventral 3 ventral 3		
	Blast	Cust	Blast	Cust	Blast	Cust	Blast	Cust	Blast	Blast	Cust
1a	19.0	<15.2	11.6	4.0	13.4	18.2	9.7	7.8	4.7	3.7	4.6
1b	17.4	17.2	10.4	6.8	19.0		7.9		4.9	3.7	—
1c	16.5	12.7	10.3	8.6	15.7		7.4		4.6	3.9	—
2	18.3	18.4	11.6	9.7	15.2	16.7	11.5	8.8	4.7	4.4	3.7
3a	17.2	17.0	12.8	9.5	19.4	14.3	8.2	9.4	5.2	4.3	4.1
3b	16.8	16.5	13.5	9.8	18.3	16.7	10.4	9.3	5.2	4.5	4.0
3c	18.7	—	19.2	—	14.4	—	11.5	—	5.3	4.1	—
4a	18.5	18.9	9.4	7.5	16.8	13.7	8.0	8.0	3.3	3.1	2.9
4b	18.7	14.5	12.4	8.6	21.0	14.1	10.0	7.5	3.8	3.0	—
4c	17.1	—	13.0	—	—	—	8.7	—	3.8	3.1	—
5a	17.2	16.5	12.9	9.0	18.8	17.2	12.0	10.4	4.3	4.0	3.5
5b	19.2	18.5	12.7	9.4	16.3	15.9	11.0	9.7	4.5	3.0	3.3
Mean	17.8	16.5	12.0	8.3	16.7	16.5	10.0	9.3	4.8	3.9	3.9
<i>n</i>	15		14		13		13		22		
<i>t</i>	1.85		4.47		0.24		0.93		2.08		
<i>P</i>	0.05-0.1		<0.01		0.8-0.9		0.3-0.4		0.05		

All data are mg dry weight

In the desmo glycogen figures there is also a wide variation between different experiments but in any one experiment there is considerably less glycogen in the gastrula than in the blastula stage. The *t* test applied to the percentage difference shows that this would appear to be significant except in the region V 2 but more data would have to be collected and the shape of the frequency distribution curve investigated before the validity could be assured of applying the *t* test to the percentage decrease instead of to the absolute decrease.

TABLE II—DISTRIBUTION OF DESMO AND LYO GLYCOCEN IN THE AXOLOTL EMBRYO

Region	Exp no	Desmo glycogen					Lyo glycogen			Total glycogen		
		Blast	Gast	Decrease %	t	P	Blast	Gast	Blast	Gast	t	P
D 1	1a	0.62	0.43	31	6.5	<0.01	13.2	14.7	13.8	15.1	0.25	0.8
	1b	0.46	0.22	52			16.7	18.1	17.1	18.3		
	2	1.13	0.70	38			14.5	18.3	15.6	19.0		
	3	1.40	0.58	59			19.0	15.5	20.4	16.1		
	Mean	0.90	0.48	45					16.7	17.1		
D 2	1a	0.27	0.19	30	5.1	0.01-0.02	11.9	—	12.2	—	1.14	0.3
	1b	0.29	0.23	21			12.3	13.3	12.6	13.6		
	2	0.58	0.40	31			9.4	6.8	10.0	7.0		
	3	0.86	0.46	47			12.9	9.0	13.8	9.3		
	Mean	0.50	0.32	32					12.2	10.0		
V 1	1a	0.32	0.17	47	10.0	<0.01	12.9	14.6	13.2	14.8	0.6	0.5-0.6
	1b	0.24	0.35	—37			15.0	14.9	15.3	15.3		
	2	1.15	0.49	57			13.5	11.7	14.7	12.2		
	3	1.23	0.75	39			18.9	15.6	19.7	16.4		
	Mean	0.76	0.44	45					15.7	14.7		
V 2	1a	0.52	0.18	65	2.34	0.1-0.2	14.1	9.3	14.6	9.3	0.21	0.8-0.9
	1b	0.26	—	—			8.3	—	8.6	—		
	2	0.62	0.52	16			7.7	8.6	8.3	9.1		
	3	0.89	0.67	25			9.9	11.2	10.8	11.9		
	Mean	0.57	0.46	35					10.6	10.2		
D 3 + V 3	1a	0.39	0.29	26	6.9	<0.01	2.74	2.87	3.13	3.16	0.46	0.6-0.7
	1b	0.28	0.23	17			—	2.95	—	2.95		
	2	0.32	0.24	25			3.80	3.78	4.12	4.02		
	3	0.29	0.19	35			3.50	3.70	3.79	3.89		
	Mean	0.32	0.24	26					3.68	3.50		

All data are mg % dry weight

## 4—DISCUSSION

We shall not present here the reasoning which led to the undertaking of this work. A full review of the arguments leading up to it will be found in the preceding paper by Waddington, Needham and Brachet (1936) \*

The determination of total glycogen in the different regions has shown that there is a definite gradient in its distribution, the concentration being highest at the animal, and lowest at the vegetal pole. It has also been shown that during the transition from the blastula to the gastrula stage there is a significant decrease in the glycogen concentration in D 2 (the organizer region), amounting to 30 %, and a possibly significant decrease in D 3 + V 3, of 12 %.

The desmo- and lyo-glycogen figures show that there is a considerable variation in the amount of desmo-glycogen which is originally present, but that during development there is a definite decrease of this component in all regions. (In V 2 this decrease is not statistically significant, but it is possible that a gross experimental error may have occurred in one of the three experiments from which *P* was evaluated.) This result agrees with that of Brachet and Needham (1935), who estimated the total and lyo-glycogen of the whole embryo of *Rana fusca* at different stages of development, using the trichloroacetic acid method, and indirectly found a decrease in the amount of desmo-glycogen with age.

From the figures given in Table II there would not appear to be a specially marked decrease in desmo-glycogen in any given region.

TABLE III

Region	Total glycogen			Desmo glycogen		
	Blast	Gastr	%	Blast	Gastr	%
D 1	17.8	16.5	7	0.90	0.48	47
D 2,	12.0	8.3	31	0.50	0.32	24
(invaginating organizer region)						
D 3 + V 3	4.3	3.9	9	0.32	0.24	26
V 1	16.7	16.5	1	0.76	0.44	42
V 2	10.0	9.3	7	0.57	0.46	19

All data are mg % dry weight

\* Since then further work on the histochemistry of glycogen in the amphibian gastrula has been published by Miyayama (1936), his paper has not been available to us. Pasteels (1936), using new histochemical methods, doubts the disappearance of the glycogen from the invaginated material in the amphibian gastrula, but such methods cannot, in the nature of the case, be decisive.

It is also clear that in that portion of the ectoderm which invaginates the glycogen which disappears is not mainly desmo glycogen for the total decrease is nearly 4 mg % whereas the greatest decrease recorded in desmo glycogen for that region is less than 0.5 mg %

The results may be briefly summarized in Table III

Our best thanks are due to Mr Marschak for assistance in the statistical treatment of the results. We make grateful acknowledgements to the Rockefeller Foundation for a grant in aid of the research expenses

#### SUMMARY

1 Total glycogen, lyo glycogen and desmo glycogen have been estimated by quantitative micro chemical methods in the amphibian embryo around the period of gastrulation

2 Before gastrulation the concentration of glycogen is found to be highest at the animal, lowest at the vegetal pole

3 During gastrulation the total glycogen decreases in amount all over the embryo but especially markedly (30%) in the material invaginating through the dorsal lip of the blastopore. Desmo glycogen also decreases in all regions but not in one region more than another. It is unlikely therefore that desmo glycogen is identical with that fraction of glycogen to which the evocator is attached in the cells

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Studies on the Nature of the Amphibian  
Organization Centre

VI—Inductions by the Evocator-Glycogen Complex in  
Intact Embryos and in Ectoderm removed from  
the Individuation-field

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[Plate 18]

I—INTRODUCTION

Several authors, including Fischer, Wehmeier and their collaborators (1933, 1935) and Waddington, Needham, Nowiński and Lemberg (1935), have obtained the induction of neural tissue in amphibian embryos by the implantation of crude glycogen or of substances extracted from it. Others (Holtfreter 1933, 1934, Woerdemann 1933) have employed it unsuccessfully. It is clear now that the activity is not due to the glycogen itself, but to some accompanying impurity, and it was suggested by Waddington, Needham and Brachet (1936) that the active evocator substance is of a sterol-like nature and forms a loose complex with glycogen and possibly with some protein as well. It therefore seemed interesting to investigate whether any difference could be detected in the evocating powers of glycogen in its two forms—the desmo-form in which it is combined with protein and the lyo-form in which it is free (Willstätter and Rohdewald 1934). Specimens of lyo- and desmo-glycogen were therefore prepared and implanted. Both showed themselves capable of evocation, and their activity was such that they seemed suited for testing the effect of a

chemically homogeneous evocating mass on ectoderm isolated from the host. By implanting desmo glycogen into isolated pieces of ectoderm we should obtain some idea of what a single chemical stimulus is capable of evoking: does it simply induce neural tissue or is there any tendency for the isolated ectoderm to react by producing a neural organ? This question was raised at the time of the discovery of the activity of the dead organizer (Waddington 1933) and has often been mentioned subsequently (in particular Waddington 1934, Needham 1936b). The present results seem to answer it in favour of the first alternative.

## 2. METHODS

Preparations of desmo- and lyo glycogen from rabbit liver were made as follows.

145 g of liver which had been cooled in ice as soon as it had been removed from the animal was minced up with scissors and added in portions to 150 ml of boiling water. When all the tissue had been added the flask was warmed for a further 20 min on a boiling water bath with occasional shaking. The mixture was then filtered on a Buchner funnel (Filtrate I). The residual tissue was ground up without sand in a mortar heated on the water bath for 20 min with a further 200 ml of water and filtered as before (Filtrate II). It was assumed that most of the lyo glycogen would have been extracted in the first two operations and most of the remainder was then removed from the tissue by extracting with boiling water five more times. 200 ml of water were used for each of these extractions which were allowed to proceed on the boiling water bath for 1-2 hr. The extract was removed by centrifugation.

The solid residue which weighed 105 g was heated on the water bath for 45 min with 200 ml of 40 % sodium hydroxide. 350 ml of 99 % alcohol were then added, the flask warmed and then allowed to stand overnight. The precipitated glycogen was washed twice with 70 % alcohol but as it was still dark and gummy it was heated for a further 45 min with 30 % potassium hydroxide. The glycogen was reprecipitated, washed once with 70 % and then three times with 99 % alcohol and dried in the desiccator for 2 days. Yield 0.68 g.

Filtrates I and II were massed (vol. 250 ml) and heated on the water bath for 15 min with 100 g of solid potassium hydroxide. 325 ml of 99 % alcohol were added and the flask was warmed then allowed to stand overnight. The precipitate was collected by centrifugation, washed with 70 % alcohol and then heated for a further 45 min with 40 % sodium

hydroxide. The glycogen was then reprecipitated, washed three times with 99% alcohol and dried in the desiccator. Yield 2.6 g.

When implantation was attempted with these samples they were found to have a destructive effect on the embryos which was traced to the presence of small amounts of sodium or potassium carbonate. This was removed by washing the glycogen with alcohol containing a trace of acetic acid—sodium acetate is soluble in alcohol—and then with pure alcohol. The material was dried in a desiccator over calcium chloride and sodium hydroxide.

The glycogen was implanted into young gastrulae of *Triton alpestris* through a slit in the blastocoele roof in the usual way. For this purpose the powder was made into a jelly by mixing with egg albumen; the mixture being then coagulated by heat. The proportion of glycogen was about 30%. In the experiments with isolated ectoderm only desmo glycogen was used, mixed with agar in the same proportions, and placed between two flaps of ectoderm (presumptive neural plate and epidermis). These explanted tissues were cultivated in Holtfreter solution and were fixed 3–5 days after operation.

### 3. EXPERIMENTAL RESULTS

As can be seen from Table I both desmo- and lyo glycogen induce neural tissue. The proportion of positive cases reckoned on the usual scale which has been employed in these papers shows a very slight advantage to the desmo fraction, and this becomes more significant if one pays attention only to the full inductions of neural tubes; thus the desmo induced five tubes in eighteen experiments, the lyo only three in twenty-four. The difference is not large, however, and is less important than the fact that lyo glycogen is active (see figs. 1 and 2, Plate 18).

TABLE I

The classification follows that of the earlier papers in this series.

Exp.	A	C	B+++	B++	B	B	D	I	Total
Exp. E 746 Desmo glycogen	5	3	0	1	1	2	5	1	18
Exp. E 766 Lyo glycogen	3	1	2	4	7	1	4	2	24
									42

The neural tubes induced by the implants have the normal appearance of tubes induced by dead material. That is to say, in the neurula stage the tube is complete in cross section but somewhat untypical in shape, often



with a circular lumen or with a slit like lumen the sides of which are unequal in thickness. It is impossible to state that the induced tube represents a definite section of the normal neural axis but we know from the work of Holtfreter (1934) and others that such tubes may later develop into recognizable if deformed parts of the neural system.

The neural tissue found in the isolated pieces of ectoderm which have been cultivated in contact with inducing material presents a totally different appearance (see figs 3 4 5 6 Plate 18). In some cases the agar glycogen evocator mass was expelled before the two pieces of ectoderm had healed together but in the other specimens an induction has occurred in nearly every case. The neural tissue is present in great quantity there is much more than would make up a piece of neural tube of normal cross section. This tissue shows extremely little sign of morphological arrangement. The elongated nuclei are indeed not disposed entirely at random for there are considerable areas in which the nuclei are parallel and the cells are elongated in the same direction so that a definite tissue not a mere disorderly mass of cells is formed. The tissues form solid masses, usually with a radial arrangement of cells and nuclei. If the masses pass a certain limit in size there becomes apparent a tendency for cavities to appear within them and the cavities of which there may be several in a single mass of tissue may be joined together to form irregular and branching tubules. There is very little sign that the walls of the tubes take up the shapes characteristic of any part of the nervous system but in some specimens a cavity may be prolonged into a comparatively small extremity of an irregularly shaped mass and in this region one can find sections showing an approximately circular piece of neural tissue containing a circular lumen.

The lack of regionalism in the induced neural tissue is further shown by the fact that no cases occur of the secondary induction of structures characteristic of particular parts of the body. As is well known an induced neural tube which has a regional structure for instance one in a normal host embryo is usually accompanied by structures such as lenses ears nasal pits etc which it has itself induced. No such structures are found in these specimens although they were certainly old enough to show them. The only kinds of secondary inductions which have occurred are two in number and both of a general nature unrestricted to particular parts of the body. One is the formation of a neat two layered ectodermal epithelium in place of the compact masses of cells which are found in isolates which do not contain neural tissue. This layer is pushed away from over the neural masses so as to form quite large cavities a repulsion which recalls the sharp separation of normal neural tube from normal overlying

ectoderm The second is the formation of melanophores above the neural tissue in some specimens Both these reactions of the ectoderm are found throughout the whole length of the neural tube in normal embryos and they are probably reactions to the presence of neural tissue as such without any reference to a particular part of the axis

#### 4—DISCUSSION

##### *Inductions by Lyo and Desmo glycogen*

As the preceding paper in this series has shown (Heatley and Lindahl) there is a quite general decrease of glycogen all over the embryo during gastrulation most severe however in the invaginating material where the primary evocator is being liberated The desmo glycogen of which there is much less decreases to about the same extent in all parts of the gastrula Now although desmo glycogen provided an analogy for the loose complex between glycogen the evocator and some protein envisaged by Waddington Needham and Brachet (1936) there was some difficulty in supposing that the desmo glycogen could itself be the fraction of glycogen in question On the one hand desmo glycogen is just that portion of the total glycogen not extractable by boiling water and on the other hand we know that boiling frees the active evocator The present results demonstrate that the evocator is associated both with the lyo and desmo fractions and suggest that if its connexion is with glycogen bound to protein the glycogen is not so strongly bound as the desmo fraction This is of course assuming that the association between evocator and glycogen is not purely fortuitous or an artifact if it were we should perhaps not expect to find the evocator attached to glycogen prepared in two quite different ways

The importance of loose complexes between proteins polysaccharides and lipins has in recent times been further appreciated Thus Posternak (1935) finds that 80 % of the phosphorus of starch can be extracted with organic solvents suggesting that its presence is due to lipoidal admixture The long series of papers by Przyłęcki and his collaborators have shown that polysaccharido proteins are readily formed and difficult to decompose considering the nature of the bonds involved (Przyłęcki and Majmin 1931 Przyłęcki and Dobrowolska 1932 Przyłęcki and Grynberg 1932 Bartuszek 1932 Przyłęcki and Bialek 1932 Przyłęcki and Fargonska 1932 Przyłęcki Mystkowski and Niklewski 1933) By adding a mixture of globulin and lecithin to a glycogen solution 80 % of the glycogen present can be adsorbed to form a complex precipitate (Przyłęcki and Majmin 1934) and complexes of proteins with cholesterol are described (Przyłęcki Hofer and

Frajberger Grynberg 1936) Such complexes as these latter are presumably responsible for the immunological phenomena following the use of sterols as antigens described, with the literature, in the book of Marrack (1934, pp 74 ff) Of special interest is the recurring observation by Przyłęcki's school that, of all the proteins, the globulins have much the most affinity for polysaccharides Here there may be a clue to the mode of action of the evocator complex for it has been suggested (Needham 1936*a* p 149) that the globulins of eggs not the phosphoproteins, are the carriers of morphogenetic polarity, symmetry, etc., if judging from what is known of myosin, we may expect that they will, when examined, turn out to have highly anisometric molecules Thus the ovoglobulin of the hen's egg has been found by Böhm and Signer (1931) to show double refraction of flow and recently Wohlsch and Belonoschkin (1936), using the test of depolarization of the Tyndall beam, find for it the highest  $\theta_0$  value yet recorded for any protein We have, of course, as yet no proof of the morphogenetic significance of thread like or rod like molecules

*Inductions by Glycogen evocator in Explanted Ectoderm*

Even before the activity of the dead organizer had been demonstrated, it had become clear that at least two aspects can be distinguished in the complex process of induction Mangold and Spemann (1927) and Spemann (1931) for amphibians and Waddington and Schmidt (1933) for the chick had drawn particular attention to the fact that a living organizer induces not merely a tissue but an organ belonging to a definite region of the body, and the latter authors had produced evidence that the region determination was a process to some extent separable from the mere guiding of the ectoderm into a path of neural differentiation The two names of "individuation" and "evocation" were suggested for these two processes (see Waddington and Needham 1936), but there has always been some doubt as to exactly where evocation stops and individuation begins The question is, If a single chemical stimulus, which must be quite without individuating action, acts on isolated competent ectoderm, just what will the ectoderm produce? It might produce either (1) a definite part of the neural tube, with "regional character" or (2) something which had a certain morphological unity and could be called a piece of neural tube belonging to no definite region or (3) masses of neural tissue, or finally (4) masses of neural cells of random orientation morphologically completely unorganized

Until now, no way of deciding between these alternatives has been available Clearly, no experiments in which evocators have been used on whole embryos can be relied upon in this connexion, since the host can

certainly exert an individuating effect on the induced neural tissue. Of experiments with explanted and isolated fragments of ectoderm Holtfreter's cultivations of fragments in contact with dead organizing tissues showed a certain rather undecided morphological organization which was certainly not characteristic of any particular part of the body and which he related to tissue movements accompanying the induction. In some of his experiments in which the ectoderm lay on the surface of dried chick embryo extract complex inductions containing notochord were obtained and although again there seems to be no evidence that the induced materials represent any definite level on the axis they certainly do not consist merely of unorganized neural tissue and seem to favour the second possibility mentioned above. However their cogency is reduced by the consideration that there is no reason to suppose that the stimulus exerted by embryo extract or dead embryonic tissue is a simple one. There is always the possibility that notochord and neural tissue represent the reaction of the ectoderm to two different stimuli and that both these stimuli are provided by the heterogeneous material to which the ectoderm was exposed.

The glycogen which was used in these experiments can probably be taken to exert a simple homogeneous stimulus. The stimulating evocator is indeed an impurity in the glycogen. It could perhaps be argued that if one impurity is present there may be more but against this there is the evidence that the reaction of the explanted ectoderm gives no hint of any heterogeneity in the stimulus which could lead to individuation. There is no trace either of the formation of a definite region of the body or even of a tendency to form a generalized embryonic axis. No tissue other than nervous tissue is formed as a direct result of the evocating stimulus and this has no further morphological organization than the radial arrangement of the third possibility. This radial arrangement is associated with the development of small irregular cavities but these do not seem to be classifiable as neural tubes in the usual sense. They suggest that the formation of cavities is a property of neural tissue as such independently of any morphological organization of a higher grade. The reaction to the evocator which we have employed is therefore clearly of the third type mentioned above.

The possibility arises however that such behaviour may not be entirely general. Lopaschov (1935, 1936) has reported that if the eyes are removed from several neurulae coagulated by heat and implanted into isolated flaps of ectoderm they may induce well shaped eyes. Lopaschov found that no definite eyes were induced if less than a certain quantity of

dead material was employed, but it does not seem possible to attribute the lack of definite organs in our specimens to a mere quantitative lack of evocator, since the masses of neural material which we obtained are very large sometimes more than half the total volume of the explant. It is just possible, though perhaps not very likely, that a certain morphological arrangement persisted in the dead material used by Lopaschov as implant, and that this is responsible for the induction of morphologically organized entities. We must, however, seriously consider Lopaschov's own suggestion, that there are specific substances to which the ectoderm can react with the formation of organs. Further work on this matter is much to be desired.

The development of a morphologically organized structure such as an eye or a definite region of the neural tube involves a co-ordinated series of foldings, thickenings, etc. and each point in the developing mass must have physical or chemical characteristics which cause it to perform exactly those changes which are required of it. It is this system of physico-chemical values which has been referred to as the individuation field. The facts of normal induction by living organizers show clearly that the individuation field of an induced neural tube can be influenced by that of the inducing organizer material: in fact the influence is usually so far reaching that the region of the induction corresponds exactly with that of the inducer. This is obviously necessarily so if, as is suggested by our experiments reported here, the competent ectoderm can of itself produce no morphological organization characteristic of a definite region. If we adopt Lopaschov's hypothesis, that induction is normally an affair of different substances which specifically induce certain organs, we must admit that the position in which these stuffs are liberated is so exactly controlled by the individuation field of the inducing tissues that the regional correspondence of inducer and induced is ensured. Thus Lopaschov's specific substances become the mechanism by which the individuation field of the inducer controls that of the induced.

The control exercised by the diffusion of one single substance can hardly be very detailed. One can, perhaps, imagine the production under the stimulus of a specific substance, of an organ symmetrical about the area from which the substance originates: one might in this way obtain the induction of a radially symmetrical eye or, if the substance diffuses out from a long narrow area, of a bilaterally symmetrical organ. But if a specific substance can really induce the formation of a perfectly shaped eye, we are set the more difficult task of finding how the ectoderm, which we know to be comparatively uniform and indifferent, can produce within itself an asymmetrical disposition of forces which can mould it into a

complicated shape The discovery that the production of an asymmetrical organ can be entirely accounted for by a reaction between a specific substance and a homogeneous ectoderm although it may appear to bring us immediately within the sphere of physico chemical concepts would actually render the process of development less and not more easy to understand in material terms

One of us (C H W) makes grateful acknowledgement to the Royal Commissioners of the Exhibition of 1851 for a Studentship held during the course of this research The thanks of the authors are due also to the Rockefeller Foundation for a grant in aid of the research expenses

#### 5—SUMMARY

1 Both desmo and lyo glycogen preparations may be used to produce successful neural inductions in the amphibian embryo Desmo glycogen cannot therefore be identical with that fraction of glycogen to which the evocator is attached in the cells

2 The distinction between evocation and individuation has been studied by implanting desmo glycogen preparations containing the evocator into isolated pieces of competent ectoderm Massive neural inductions are produced but these have not the character either of recognizable parts of the nervous system or of sections of a normal neural tube On the other hand the cells are not oriented entirely at random They tend to arrange themselves radially surrounding hollow tubular spaces of which there may be as many as six branching or independent in one induction This is believed to demonstrate the effect of the homogeneous stimulus of the primary evocator

3 Following on the induction of neural tissue the repulsion of overlying ectoderm and the formation of melanophores were observed These two effects are interpreted as dependent differentiations brought about by the presence of neural tissue as such

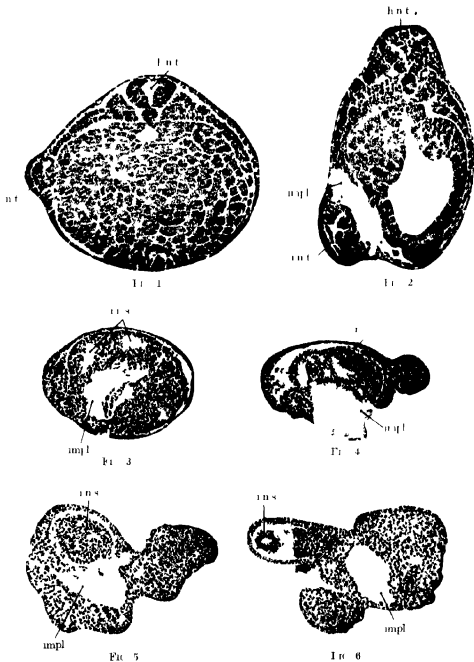
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# DESCRIPTION OF PLATE 18

- FIG 1, No E 74b-3—Secondary neural tube induced by desmo glycogen  
 FIG 2, No E 76b-5—Secondary neural tube induced by lyo-glycogen  
 FIG 3, No E 131d-5—Neural tissue induced by desmo-glycogen in isolated ectoderm of axolotl Fixed after 4 days  
 FIG 4, No E 131d-9—The same Fixed after 4 days  
 FIG 5, No E 131d-12—The same Fixed after 4 days  
 FIG 6, No E 131d-20—The same Fixed after 5 days



*hnt* host neural tube *ins* induced neural structures *impl* implant





# On the Change over in the Oestrous Cycle in Animals after Transference across the Equator, with Further Observations on the Incidence of the Breeding Seasons and the Factors controlling Sexual Periodicity

By F. H. A. MARSHALL, F.R.S., Cambridge

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In the Croonian Lecture (1936) I adopted the view that sexual periodicity is primarily a function of the gonads which show an alternation between a condition of activity and one of rest, but that the reproductive rhythm, while metabolically conditioned and to some extent directly controlled by the general environment, is in the higher forms of life regulated further by exteroceptive stimuli conveyed by nervous paths to the hypothalamus and thence transmitted to the anterior pituitary. The evidence for this view is there summarized, and it becomes apparent that in mammals and birds at any rate the nervous system plays an important part in the regulation of the oestrous cycle.

In a large number of animals the incidence of daylight is almost certainly an important factor in controlling the cycle. The ruminants, however, are exceptional since if they react to light at all it must be to diminution rather than to increase. That they react to seasonal change in a remarkable way is shown especially by those natural experiments in which individual animals of varieties or species that normally breed once annually, have crossed the equator and as a consequence have been induced to have two sexual seasons in one year. At any rate these cases cannot be explained simply as due to an endocrine rhythm.

## SHEEP

The case of the flock of twenty-one Southdown ewes (as described by Dr. L. L. Roux) which were imported when pregnant into Cape Province, South Africa, has already been recorded in a footnote to the Croonian Lecture. The ewes lambed in January 1933. They came on heat and were tupped in May 1933 so that the change over to conform to the sexual season of the southern hemisphere was at the beginning very rapid. In

1934 they came on heat in April, and in 1935 and 1936 they came on heat in March which is nearly the reverse of the normal tuppung time in England. It is thus seen that in just over 2 years they became almost or quite adjusted to the South African seasons.

A second case of interest is one of twenty-five Southdown ewes which were imported to the Argentine from England in April 1935 and kept on the estate of Sucesion Julio Pueyrredón El Campamento, Nahuel Ruca, F C S. The ram was put in with them in December 1935 at a time when they would have been breeding if in England. Only three of the ewes produced lambs. It is evident that the English sexual rhythm had been disturbed by the ewes having been transferred to a country with reversed seasons.

In a third case a batch of Southdown ewes was imported to the Argentine from England in April 1936, and kept by Sucesion Pueyrredón on the same estate. The ram was put with them in July, that is, at a time when Southdown sheep do not breed in England. All the ewes were duly served. It is apparent that the sexual season was accelerated.

For the information about the last two cases I am indebted to Dr John Hammond who visited the Argentine in the second half of 1936. As is well known Southdown sheep in England have only one breeding season (in the late summer and autumn).

In partial confirmation of the above recorded results I am informed that pregnant ewes imported into Austraha after lambing at the English time will often take the ram (shortly after parturition) at the Australian time and thereafter conform to the Australian seasons. My information comes from Major Robert A. Wilson who obtained it from Mr Fawkes, the statement being based on Mr Fawkes's own experience. I am not, however, in possession of exact dates.

In England, as is well known, sheep of most breeds are capable of having an extended sexual season from August to February with a succession of dioestrous cycles (leaving out of account the exceptional breeds—Dorset Horns and Merinos—which can have lambs twice a year). In Scotland the tuppung time is later (October to December) especially in the Highlands, and speaking generally is more limited in duration the farther north we go. This may be partly a breed difference but it is certainly not entirely so since Scottish Black faced sheep are known to breed later and to have fewer dioestrous cycles (in the absence of the ram) in the Highlands as compared with the Lowlands. Moreover, Mr Halldór Pálsson informs me that with the native Icelandic sheep, the ewes come on heat in mid November and are usually tupped between Christmas and the New Year. The lambs breed in December when 7 months old. Furthermore, Dr

Hammond ascertained that in the extreme south of the Argentine (in Patagonia) the sexual season of the Romney Marsh sheep is very limited there being only two or three dioestrous cycles in May and June as with Scottish sheep in the Highlands in November and December. On the other hand Dr Hammond found that sheep of the same breed in the north of the Argentine (Province of Buenos Aires) have an extended sexual season from December to June comparable to what they experience in the reverse time in Central and Southern Europe.

The above recorded facts do not admit of a complete interpretation for the recurrence of the sexual seasons cannot be simply explained as the result of a response to diminution of daylight. Nevertheless there is evidently some correspondence between the oestrous cycles of the sheep and the seasonal environmental conditions as experienced in different parts of the world in both hemispheres. This is shown in the existence of a relation (but not a very close one) between the latitudes and the times of breeding.

#### RED DEER

Red deer have been imported into New Zealand from Scotland and England as well as from various countries in Europe and wherever they came from they have adapted themselves to the southern hemisphere seasonal conditions. Thus with the stags the times for rut and for the growth and shedding of the antlers and pelage are the reverse of the times in Europe and with the hinds the periods of oestrus and of parturition are also in actual time reversed. Donne (1924) who had access to the State records of New Zealand has supplied some information as to the change over in individual deer. He states that two stags and four hinds (all 4 years old) were shipped for New Zealand from Warnham Court Park, England on 4 October 1907. Two of the hinds died on the voyage but the rest reached New Zealand in mid November and were placed on the Paraparaumu game farm. The stags came out of the bush with clean antlers and began roaring on 2 May 1908, 2 or 3 weeks after the New Zealand stags had ceased rutting and about 6 weeks after they began. These stags therefore had shed their antlers and grown new ones twice in one year but the first antlers grown in New Zealand had apparently not had time to develop fully being smaller than those which were shed. Thus under exceptional circumstances stags may rut without the antlers having reached the full normal growth for animals of the age in question. In 1909 the stags rutted at the end of March at the usual time for New Zealand.

The two surviving hinds which were pregnant to stags in England calved in April 1908 at the normal English time. Donne states that they calved again in February 1909. Assuming that this is correct they must have become pregnant to stags which were rutting in the previous July, which is an unusual time for New Zealand. Donne says that "in 1910 they had adapted themselves to local conditions". It would thus appear that stags may take about 18 months or less to become adjusted to the southern hemisphere seasonal conditions after importation from England and that the oestrous cycle of the hinds is fully reversed after about 2 years.

Mr Conrad Hodgkinson of Otago has kindly supplied me with confirmatory information in regard to the change over in stags. He states that two animals (both 2 years old) were imported into New Zealand in the 'late fall'. Instead of shedding their hair and horns they grew a thick shaggy coat and retained their horns for some months. In the following autumn (12 months after their arrival) one of them took a small herd of hinds and 'was undoubtedly on the rut'. It must be understood that it was not easy to observe exactly what happened to deer after they were turned out as often they were not seen again for some time. Mr Hodgkinson reports another case of a stag, imported in the New Zealand spring, which took up with some hinds when turned out later, and rutted normally (holding seven hinds) 18 months after arrival.

Through the kindness of Mr C. E. Lucas of Warnham Court, Sussex, I have obtained a record of the sexual seasons of the New Zealand deer which were imported to Warnham Park in the late autumn of 1910. There were eight hinds, all of them yearlings or rising 2 years old. They first came on heat in April 1911 which is the normal time for red deer in New Zealand. One or two of the Warnham stags were seen to cover the hinds but there were no calves. Mr Lucas thinks that the non occurrence of pregnancy may have been due to the hinds being yearlings. The fact that the hinds' first oestrus in England was at the New Zealand time shows that at this phase the original oestrous rhythm was a stronger factor in the control of the cycle than any external causes. The hinds next came on heat in December-January (1911-12) and Mr Lucas himself saw one of the Warnham stags jump a hind on 16 January 1912. Four or five very late calves were produced by the hinds but they did not survive for long although one stag calf and one hind calf lived through the winter of 1912-13. The New Zealand hinds became fully acclimatized by the autumn of 1912 and experienced oestrus at the normal English time. Thus, it took approximately 2 years for the imported deer to adapt their oestrous cycles to the English seasons.

It has been mentioned that Warnham stags were seen to copulate with the New Zealand hinds in April and again in December and January, these months being outside the ordinary rutting time for stags in Great Britain. The rut was observed by Mr Lucas as well as by the late Mr J G Millais who was a neighbour of Mr Lucas and a close observer of the Warnham Park deer. Mr Millais reported the occurrence to Mr Donne stating that "when the New Zealand hinds came into season [in April] the whole of the stags in Warnham Court Park began roaring, fighting and carrying on in a wild crazy manner and rutted, although many of them had shed one or both antlers, and all of them had participated in the local rut during the previous September and October." Millais expressed the view that it is the hinds more than the stags which are responsible for the time of rut and cited this incident as evidence (Donne 1924). \* Dr Fraser Darling who has made a close and extended study of the red deer on Priest Island, Wester Ross, Scotland informs me however, that the testes of the stag become very perceptibly reduced in size after the normal rut is over (in December) and presumably go into a state of relative quiescence. Very exceptionally, he says, a hind may be rendered pregnant by copulation in February but that when this happens it is due to service by a young stag which had not mated during the regular season. With park stags in the south of England the capacity for breeding may perhaps be greater and it is probable that the level of their testicular activity does not fall so low that it cannot be raised by exteroceptive stimuli such as those resulting from the proximity of hinds in a stage of oestrus.

It has been said that if the sexual cycle in deer is related at all to daylight it must be to diminution of light. It is possible however, that temperature may be a contributory factor for there is a common belief which is well attested that in Scottish deer general rut does not occur properly until there has been a sharp frost that is to say, that a frost will awake both stags and hinds to their full sexual activity. Major R A Wilson has made an identical statement about red deer in New Zealand, basing it on his own observations. Furthermore, Lord Latymer informs me that in his own experience in Scotland "if there is an exceptionally mild autumn, such as occurs only once or twice in a half century there is very little mating, and very few calves will be dropped in the following spring." In experiments on sheep, however, McKenzie and Phillips (1933) have shown that there is no significant difference in the times of coming on heat when the

\* Mr Lucas informs me that Donne is incorrect in stating that this rut resulted in one of the New Zealand hinds bearing a calf. The stag with exceptionally fine antlers to which Donne refers was conceived at a later rut.

ewes are subjected to different degrees of temperature for some time before the beginning of the sexual season, a low temperature having no effect in accelerating the cycle

#### OTHER RUMINANTS

Of the other ruminants which have been introduced into New Zealand all have reversed their actual times for breeding. Thus, Donne (1924) states that the fallow deer, which ruts in England in October, 2 or 3 weeks later than the red deer, in New Zealand ruts in April, the moose, which has its sexual season in September and October in Canada (a little later than in U S A ) in New Zealand ruts in March and April, the wapiti ruts in New Zealand from the third week in March until mid April, the reverse of the Canadian time, and the Virginian deer which in America has its season in October, in New Zealand 'should rut' from April to early June (Donne 1924). Lord Latymer informs me that the chamois, which in Europe mates in mid November, has also reversed its time in New Zealand.

The case of the Thar is especially interesting. In India it ruts in September–October but in New Zealand in April–May (Donne 1924). Dr Zuckerman informs me that for this species in the Zoological Society's Gardens in London, the breeding season gradually became later, the peak of births (as shown in frequency curves which he constructed from the Society's records), during the period 1891–1910 was in May but in the next 20 year period 1911–30 it was in the first part of July (see Marshall 1936). Since the period of gestation in the Thar is 8 months the corresponding sexual seasons for this species were respectively September and early November. It is thus seen that the New Zealand sexual season is the reverse of the *acquired* season in England and about 7 months later (or 5 months earlier) than the time of rut in India.

There is no information available for any of these species as to the process of reversal in particular imported individuals.

Of the domesticated ruminants introduced into the southern hemisphere goats, like sheep, reverse their breeding seasons so as to conform to southern conditions. In England the more active season is in the second half of the year but conception *may* take place in any month. October is the most frequent month and May the least frequent (Asdell 1929). Swiss goats usually breed from October to January. In South Africa Boer goats breed from April to August and Angora goats from February to August (Kupfer 1928). Thomson (1922) states that the "wild goats" at Skippers, New Zealand, have their young "in winter", and Zuckerman (1932), who

quotes this observation, says it suggests ' that the new environment has not altered the goat's European breeding habit" This explanation, however, is not valid since goats frequently breed in England in September and produce kids in February

Of the native ruminants in the southern hemisphere it is to be noted that all those species which live some distance from the equator appear to rut in the first part of the year (late summer or autumn) This is the case with the white tailed and brindled gnus the steenbok, impala, spring buck, bush buck and inyala The sexual season of the graffe in South Africa is from August to December Tropical and some subtropical antelopes which live under comparatively uniform conditions as regards daylight may have extended breeding seasons or breed all the year round, and the red and blue duikers also have variable or extended breeding seasons as likewise, do the roan and sable antelopes and the kudu and the eland (Fitzsimons 1919-20) In the Cape buffalo the sexual season is April to June according to Lydekker (1898*b*), but it is variable according to Fitzsimons (1919-20)

South American deer appear to breed in the autumn Thus, the red Brocket (*Mazama rufa*) is said to have a sexual season in April (Lydekker 1898*a*)

All the northern deer rut in the autumn as do also the prongbuck, the Indian buffalo, the musk ox, and the various species of sheep and goats The American and European bison rut in the late summer or autumn The Alpine and Asiatic ibexes however are stated to have their sexual seasons in the winter (Lydekker 1898*b*) Lydekker (1924) mentions a few other species which are stated to rut at unusual times for ruminants Thus, the muntjac is described as rutting in January to February or irregularly, the musk deer in January, the *Memima chervrotain* in June, and the *Thamin* from March to May The black buck or Indian antelope is said to rut in February or March but nevertheless young may be seen at any time (Lydekker 1924), so the duration of its rutting season must be regarded as doubtful

Tropical and subtropical deer, like antelopes, tend to have extended sexual seasons and may breed at any time of the year This is the case for example with the axis Moreover, according to the Marquess of Tavistock, no tropical deer adapt themselves to the English seasons According to Donne (1924), however, the sambar introduced into New Zealand, ruts in March and April

Some domesticated ruminants as with domesticated mammals of other species (e.g. dogs, cats and rabbits) seem to have partly or entirely freed themselves from the influence of seasonal exteroceptive stimuli This may



be partly due to the fact that for many generations they have not been under the influence of natural selection the capacity for response being of little or no biological advantage to them Thus domestic cattle may breed at any season of the year With wild white cattle there is however a tendency to calve in the spring as a consequence of mating about July (Wallace 1907) Moreover Roberts (1928) states that with domestic cows as age advances an increasing proportion calve in March April and May and at the sixth time of calving this proportion is nearly 50 %

It is thus seen that ruminants with very few exceptions have their sexual season in the autumn and so differ from the majority of animals which breed in the spring Seeing that the gestation period in ruminants is usually some 5-8 months it is no doubt true that teleologically speaking the times of rut are so arranged that the young shall be born at the most favourable season for their development It might be argued that the time of parturition has become fixed by natural selection and that the rest of the sexual cycle has become arranged around this point the successive stages depending merely upon a natural rhythm This explanation however is not entirely satisfactory since the period of gestation being generally fixed within rather narrow limits for each species the time of parturition depends upon the time of oestrus and rut and where this is at an abnormal season (as in exceptional cases) the time of parturition is also correspondingly abnormal The physiological problem as to the causes of the seasons in the individual animal is different from the teleological one and in considering these causes it becomes apparent that the point at which the cycle is brought more particularly into relation with the seasonal environment must be the time of rut and oestrus upon which in each species the rest of the cycle mainly depends As already indicated diminution in daylight has been suggested as a possible stimulus and temperature is sometimes perhaps a contributory factor as has been supposed for deer Indeed it would appear likely that there is a combination of stimuli which judging by analogy from what is known concerning other animals are exteroceptive in character Tropical ruminants when brought to temperate countries do not usually adjust themselves to the changing seasons because they do not have the capacity to respond to appropriate stimuli so as to undergo rut or oestrus at fixed times of the year

That the degree of reaction may vary with allied genera must also be noted Thus the reed buck and the bush buck inhabit the same part of South Africa and are exposed to the same conditions yet the reed buck may breed throughout the year whereas the bush buck as already mentioned has a restricted season (Zuckerman 1932)

## OTHER MAMMALS

A comprehensive comparative account of the seasons for breeding in all mammals would require a whole volume. Here it may be said that the insectivores, carnivores, rodents and non-ruminating ungulates with very few exceptions outside of tropical areas breed in the spring or first half of the year in the northern hemisphere and in the spring or second half of the year in the southern hemisphere. In subtropical areas there may or may not be a breeding season with the mammals inhabiting them, and its occurrence is to some extent related to latitude. Some of the rodents in temperate countries are exceptional in having very extended seasons or breeding throughout practically the whole year. Amongst carnivores the badger is exceptional in breeding in October and this may be in some way correlated with its unusually long period of gestation. (For details for different species, see Heape 1900, Seton 1909, Fitzsimons 1919-20, Marshall 1922, Wood Jones 1923-5 and Zuckerman 1932.)

Sexual periodicity in the bats has been recently discussed and the main facts summarized by Baker and Baker (1936) and Baker and Bird (1936). The fruit bats breed in the autumn (or corresponding time even though the environmental conditions are almost uniform over the whole year) in both hemispheres. The insectivorous bats that hibernate, as is well known, copulate in the autumn and ovulate in the spring, but many copulate again in the spring. The insectivorous bat *Miniopterus* in the New Hebrides has a sexual season in the southern "spring".

With marsupials nearly every month of the year may be the breeding season of some species, but many of them live under comparatively uniform conditions as regards the duration of daylight (Wood Jones 1923-5).

For the primates the evidence as to sexual periodicity has been summarized by Zuckerman (1932). Although some species are described as having restricted seasons Zuckerman concludes that, speaking generally, "monkeys and apes, like man, experience a smooth and uninterrupted sexual and reproductive life." This conclusion, however, must be accepted with reservation, and there is evidence that primitive man had an annual breeding season in spring (see Marshall 1922).

It has been already remarked that several, at least of the domestic mammals have partially or entirely freed themselves from the influence of seasonal changes upon their sexual activity, their oestrous cycles being accelerated. This is perhaps partly the result of artificial selection, but warmth, plentiful nutrition and comparatively uniform conditions are

probably contributory causes. The domestic bitch may experience oestrus at any time of the year though maintaining a definite internal rhythm. Heape (1900), however, states that in Danish Greenland the dogs have only one annual breeding season (instead of two) and resemble the wolf and jackal which in their wild state breed in the first part of the year. The dingo of Australia, which is believed to be descended from some dog- or wolf like animal, usually breeds in August and September in the southern spring (Wood Jones 1923-5). The wild cat in Britain usually breeds only in the spring (Mills 1904-6), and the allied African wild cat has its sexual season in South Africa in the southern spring (Fitzsimons 1919-20). The domestic cat, on the other hand, has two (sometimes three) sexual seasons in the year, breeding at any time though perhaps oftener in the spring.

The wild rabbit in England breeds usually from February to May but may breed again about harvest time in August. The domesticated rabbit if kept warm and well fed may breed all the year round. Mr W. Weatherly informs me that in Victoria the sexual season of the introduced rabbits is from August to October but that there may be a less pronounced season after the rains in April. In Central Australia in favourable seasons the rabbits may breed all the year round. It is said also that in New Zealand, where the rabbit has likewise been introduced, it breeds in some localities all the year. Other introduced mammals in New Zealand, excepting the ruminants, are said to breed in the southern spring.

Hammond's observations on the breeding seasons of the horse have been briefly recorded in the Croonian Lecture (Marshall 1936). In the more improved breeds especially the times of breeding are very extended but the numbers of mares served in spring as compared with other times are markedly greater, and this applies to the southern spring as well as the northern. In South Africa both for donkeys and horses there is an inactive season which ends about September (Kupfer 1928).

It is thus seen that ruminants in usually breeding in the autumn stand out in contrast to the majority of other mammals.

## BIRDS

The subject of photoperiodicity in birds has been well treated by Rowan (1926). As is well known most birds breed in the spring when the daylight is increasing and this fact was made the basis of the view which led Rowan to carry out his famous experiments on the effects of irradiation upon the reproductive organs and upon the migratory movements of birds. The native birds of the southern hemisphere (at any rate below subtropical

latitudes) are for the most part no exceptions to the general rule and it is interesting to note that according to Hutton (1901) the European species which have been introduced into New Zealand conform to the seasonal cycle of that country in regard both to the plumage changes and to their breeding periodicity. Hutton mentions the starling the linnet the redpoll the robin and the hedge sparrow as instances. Thomson (1922) says that the domestic sparrow breeds from September to April and the thrush in September and October and usually again later. He says also that the thrush in the South Island at least commences to sing in May at the beginning of the winter. Nothing however appears to have been recorded as to the change over in particular individual birds.

Of birds which have been transported from the southern to the northern hemisphere and kept in captivity the Marquess of Tavistock found that whereas parakeets from Southern and Central Australia adapt themselves to the English seasons at once and breed in spring birds from North Australia (the hooded parakeet and Brown's parakeet) retain their original breeding season which is in October. Lord Tavistock informs me further that the roseate cockatoo adapts itself at once but that the Banksian cockatoo in England is rather unadaptable usually moulting in spring and laying from late summer well into the winter. Mathews (1910-27) states that the roseate cockatoo in Australia breeds in September and November (spring) in the east and in February and March in the north west where conditions are presumably more uniform. The Banksian cockatoo breeds in May to July in Australia (Mathews 1910-27) a fact which would suggest that this species to a certain extent at any rate adapts itself in England.

According to information kindly supplied by Mr. Hugh Wormald of East Dereham the Australian maned goose or wood duck (*Chenonetta jubata*) kept by him in Norfolk laid eggs which hatched in May and this happened again in August. Mathews (1910-27) states that the breeding season of this bird in Australia is from August to January or later but in June in the north. It would seem therefore that this species in England responds to the northern hemisphere conditions breeding in the warmer and lighter part of the year.

Witschi (1935) records that tropical African weaver finches kept for 3 years in the animal room at Iowa City under constant food conditions maintained their African cycles. Their breeding season is in the fall. If light has any influence then this group of birds responds to shortening and not to lengthening of the day. Even juvenile paradise whydahs which came into their first breeding period only after they had lived one full year

in Iowa unhesitatingly fell into line with their adult companions, coming in breeding condition in August-September of their second year "

Rowan (1926) has discussed at length the difficult question as to the factors which control breeding in the transequatorial migrants and has given a critical summary of the evidence bearing upon this problem With a few doubtful exceptions these northern breeding birds do not ever breed in the south in response to daylight conditions there \* The case of the European white storks which he quotes on the authority of Murphy (1925) as having bred in captivity in Peru is one of exceptional interest For the stork is a migrating bird which crosses the equator and migrates far south annually Yet Murphy states that the storks kept in captivity at Lima bred at their appropriate season for the southern hemisphere, the sexual cycle in these birds being completely reversed †

#### DISCUSSION

The evidence here collected, which is supplemental to that presented in the Crooman Lecture, shows that the gonadal endocrine rhythm with the higher animals although sometimes maintained in abnormal surroundings (as in some species of birds), is in most species readily altered by exteroceptive stimuli or other environmental influences, and even to such an extent that the individuals of a species may have two breeding seasons in a year when they normally would have only one Moreover, considered in the light of the evidence previously brought together there is a presumption that the adjustment to the new seasonal influence is effected through the hypothalamo hypophyseal mechanism, the anterior pituitary being the principal regulator Although whole groups of animals such as the ruminants appear to react to the seasonal environment in much the same kind of way, stress must be laid on the fact that there are also wide differences in the degrees and modes of reaction (and sometimes even among allied species) This has also been emphasized by Rowan (1936) who points out that whereas ferrets have been shown to react to green light (Marshall and Bowden 1934) starlings completely failed so to react (Bissonette 1931) Furthermore, some animals are exceptional in seeming

\* The mutton bird of New Zealand and several other species of shearwaters (*Puffinus*) in the southern hemisphere do the converse of this, breeding in the south (South America, Australia and New Zealand) in the second half of the year and migrating across the equator to the north (where they do not breed) in the first part of the year (Murphy 1936)

† Concerning these birds Dr Murphy informs me that they were "courting in September, building nests and laying eggs in October, and subsequently rearing young"

not to react to irradiation of any kind as regards the incidence of their sexual season

*Experiments with Light and Ultra violet Irradiation*

There is already a long list of animals and birds which have been shown experimentally to respond to light irradiation by coming into sexual activity outside of the normal season and to this list there must now be added the white-footed mouse (Whitaker 1936) and the raccoon (Bissonette and Czech 1937). Experimental results have shown further that ultra violet irradiation may have an even greater influence in the physiological control of oestrus than ordinary light rays since its effects are more long continued. Thus, Bowden and I found that with female ferrets treated with ordinary electric light or with rays from the visible part of the spectrum in midwinter daily from about dusk to midnight oestrus supervened in about 3 weeks, but the ferrets in the following spring passed into a state of anoestrus, though they frequently came on heat again a second time later in the season after the manner of normal ferrets. On the other hand, ferrets treated for similar lengths of time with ultra violet rays came on heat in winter after a like interval but remained on heat for a far longer duration and sometimes until the end of August (Marshall and Bowden 1934, 1936). In the second year some of the ferrets treated with ultra violet rays died from uncertain causes during the late spring but they had a continuous oestrus until death. In a third year (1936) six ferrets treated with electric light in various ways behaved similarly, but the two ferrets which were subjected to ultra violet irradiation came on heat in February (a little later than the others) and remained on continuously until the end of August and then went into anoestrus. The irradiation in every case was discontinued in May. Thus, exposure to ultra violet rays caused a far more decided and prolonged effect than exposure to increased illumination. Bissonette (1936) states that the anterior pituitaries of stimulated ferrets undergo histological changes comparable to those of castrated animals, large clear cells making their appearance, and this condition has been interpreted as one of hyper-pituitarism, at least in respect of the production of the gonadotropic hormone. The excessive fertility of the marmosets at the Lister Institute which were subjected to ultra violet treatment as described by Miss Margaret Hume, is also suggestive of hyper-activity on the part of the anterior pituitary (see Marshall 1936). It may not unreasonably be concluded, therefore, that the state of long continued oestrus in the ferrets following upon ultra violet irradiation was similarly one of hyper-pituitarism. This conclusion may be of far reaching application

although as already remarked it is unsafe to argue from one species to another without obtaining experimental verification. Moreover, it seems clear that there are many species to which in a state of nature this principle cannot apply.

I have pleasure in expressing my indebtedness to the following gentlemen who have been so kind as to supply me with records or other information: The Marquess of Tavistock, Lord Latymer, Mr C E Lucas, Mr M A C Hinton, Dr L L Roux, Major Robert A Wilson, Mr Conrad D Hodgkinson, Sucesion J Pueyrredón, Professor W Rowan, Dr F Fraser Darling, Dr S Zuckerman, Mr Halldór Pálsson, Mr Hugh Wormald, Mr Mark Johnson, Mr W Weatherly, and, in particular, Dr John Hammond.

#### SUMMARY

It has been shown that Southdown sheep and also red deer after being transported across the equator from one hemisphere to the other reverse their breeding seasons so as to conform to the conditions of the countries into which they have been introduced. This statement applies not only to the breed or species but to individual animals. The change over may begin at once so that the animals can have two rutting or sexual seasons in one year when they would otherwise have only one but the complete adjustment may take about 2 years. This applies both to sheep and to deer. It is clear, therefore, that the oestrous cycle and its successive phases are not regulated solely by the endocrine mechanisms of the gonads and the pituitary, but are controlled also by exteroceptive or other external factors which probably act upon the hypothalamo hypophysial mechanism through the nervous system.

With other species of ruminants introduced into New Zealand the breeding seasons have also been reversed so as to conform to southern hemisphere conditions but no information is available as to the change over in particular individuals.

It is pointed out that with the majority of ruminants both in the northern and in the southern hemisphere (and at a considerable distance from the equator) the sexual season is in the autumn so that if these animals react to light it must be to diminution and not to increase.

Tropical or subtropical ruminants generally have an irregular or more or less continuous breeding season and do not usually adjust themselves to the changing conditions of temperate countries in regard to sexual activity.

The majority of other mammals (especially insectivores, carnivores, rodents and non-ruminating ungulates) breed in the first half of the year in the northern hemisphere and in the second half in the southern hemisphere (excepting in some tropical and subtropical countries). Species which have been introduced from the northern to the southern hemisphere conform to the seasonal conditions of the south.

With various species of birds which have been transported from the southern to the northern hemisphere the breeding season generally becomes adjusted to the new conditions but this is not always so for a few species tend to retain the cycles of their native countries. Species which have been introduced from the north to the south so far as recorded always adjust themselves.

Emphasis is laid on the fact that whereas with most animals light appears to enhance sexual activity there is a great variation in the reactions of different species.

In the case of the ferret it is shown that ultra violet irradiation has a far more extended effect upon the sexual organs than ordinary light rays. This effect must be interpreted as one of hyperpituitarism (at least in regard to the gonadotropic function) and though the principle involved may have far reaching applications it is unsafe to generalize from results obtained from one species of animal.

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## The Carcinogenic Action of Dibenzcarbazoles

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*(Communicated by E. L. Kennaway, F.R.S. — Received 10 November 1936)*

[Plates 19-22]

The known carcinogenic hydrocarbons such as 1 2 5 6 dibenzanthracene usually produce primary malignant changes at the site of application. If they are painted on the skin, papillomata or epitheliomata are produced, if injected subcutaneously sarcomata are formed. The occupational cancers of the skin no doubt depend upon local action of the same kind, but the cancer of the bladder, which is found among chemical workers, arises in an organ which cannot come in direct contact with any external carcinogenic chemical agent.

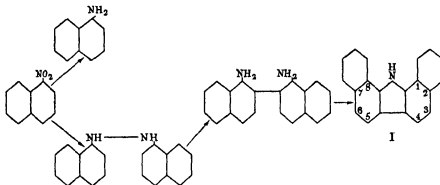
Cancer of the bladder is more frequent among operatives in chemical works than in the general population, those concerned with the manufacture of naphthylamines and benzidine seem to be most liable to this form of cancer (cf. Berenblum 1932, Hueper 1934). Tests of the carcinogenic activity of naphthylamines themselves, however, have given contradictory and unsatisfactory results and they do not produce cancer in animals when applied to the skin.

In the light of the work of Barry Cook, Haslewood, Hewett, Hieger and Kennaway (1935), which had shown that dibenzacridines were carcinogenic, it seemed possible that some impurity in naphthylamines formed during their manufacture might be the responsible carcinogenic agent.

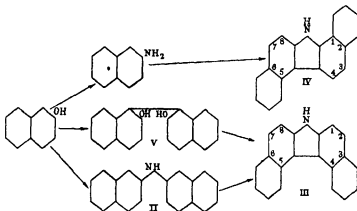
$\alpha$  Naphthylamine is manufactured by reducing  $\alpha$  nitronaphthalene with iron and a small quantity of hydrochloric acid. It is possible that under the conditions used some hydrazonaphthalene might be formed. Hydrazonaphthalene in presence of acid has been shown to undergo a benzidine change to give two products one of which can undergo condensation to form 1 2 7 8 dibenzcarbazole (I) (Vesely 1905). If the nitronaphthalene

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contained any  $\beta$  nitronaphthalene that might undergo a similar change to form 3 4 5 6 dibenzcarbazole (cf Corbellini and Marconi 1932)



$\beta$  Naphthylamine is manufactured by heating  $\beta$  naphthol with ammonium sulphite and ammonia under pressure at 150° C. It is known that excess heating or ineffective stirring leads to formation of  $\beta\beta$  dinaphthylamine (II) so that this is a contaminant of the crude product which is then distilled *in vacuo*. During the distillation it is possible that 3 4 5 6 dibenzcarbazole (III) might be formed as carbazole is formed in the distillation of diphenylamine. The presence of  $\alpha$  naphthol might lead to the formation of  $\alpha\beta$  dinaphthylamine and that in its turn to 1 2 5 6 dibenzcarbazole (IV). A probable contaminant of  $\beta$  naphthol is  $\beta\beta$  dihydroxy  $\alpha\alpha$  dinaphthyl (V) which heated with zinc ammonium chloride yields 3 4 5 6 dibenzcarbazole (III) (cf Walder 1882).



It therefore seemed possible that naphthylamine workers might come in contact with dinaphthylamines and dibenzcarbazoles and for this reason

experiments were made with benzene solutions of  $\alpha\alpha$  dinaphthylamine  $\beta\beta$  dinaphthylamine (II) 1 2 5 6 dibenzcarbazole (IV) 1 2 7 8 dibenzcarbazole (I) and 3 4 5 6 dibenzcarbazole (III) which were applied to the skin of mice. The three dibenzcarbazoles were chosen on account of their structural resemblance to 1 2 5 6 dibenzacridine and 3 4 5 6 dibenzacridine which like the related hydrocarbons 1 2 5 6 dibenzanthracene and 1 2 ~ 8 dibenzanthracene have carcinogenic properties (Cook Hieger Kennaway and Mayneord 1932 Barry and others 1935). Winterstein Schon and Vetter (1934) suggested that 1 2 benzcarbazole on the ground of its structural analogy to 1 2 benzanthracene might be carcinogenic and in a later paper Schurch and Winterstein (1935) reported that they had produced tumours of the skin in mice with 1 2 benzcarbazole.

Mice were also painted with *o* aminoazotoluene which has been shown to produce hepatoma (Nishiyama 1935) and diaminoazobenzene or chrysoidine a commercial cotton dye related to aminoazotoluene. The crystal structure of the three isomeric dibenzcarbazoles which were used has been examined by Iball (1936).

#### EXPERIMENTAL

Mixed stock mice and albino mice of the Simpson strain were painted twice weekly with 0.3% benzene solutions of the substances. Of the sixty mice painted with 3 4 5 6 dibenzcarbazole two showed papillomata after 100 days treatment but unfortunately this compound was toxic so that after 100 days treatment only one third of the original mice were alive. On account of the toxic effect of the 3 4 5 6 dibenzcarbazole the 0.3% solution was discontinued after 125 days and a 0.1% solution substituted and other series of mice were treated through out with the diluted solution. Of the mice treated with this compound eight have developed epitheliomata and four papillomata. The survival and occurrence of the skin changes in these mice is shown in figs 1 and 2. No mice treated with 3 4 5 6-dibenzcarbazole have lived more than 321 days. A typical mouse from the first group of animals treated with a 0.3% solution of 3 4 5 6 dibenzcarbazole for 125 days and then with a 0.1% solution for 111 days is shown in fig 5 Plate 19. This mouse had an epithelioma a section of which is shown in fig 6 Plate 19 with metastasis in the axilla (section shown in fig 7 Plate 19) in addition to hepatic and biliary hypertrophy.

The other compounds tested were much less toxic while 1 2 5 6 dibenzcarbazole is carcinogenic 1 2 ~ 8 dibenzcarbazole is only feebly active and the dinaphthylamines are not carcinogenic to the skin. After

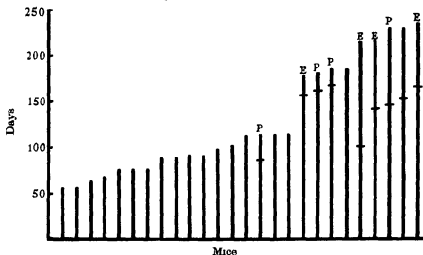


FIG 1—Mice painted with 0.3% solution of 3,4,5,6-dibenzocarbazole in benzene for 125 days and then with 0.1% solution. Thirty-four mice which survived less than 50 days are not represented. In this and in figs 2-4 each vertical line represents the duration of life of one animal after the commencement of treatment. The transverse mark on some of the lines denotes the day when a tumour was first noticed. *E*=epithelioma, *P*=papilloma

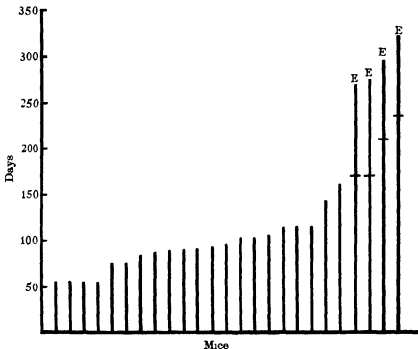


FIG 2—Mice painted with 0.1% 3,4,5,6-dibenzocarbazole. Five mice which survived less than 50 days are not represented.

340 days' treatment 1 2 5 6 dibenzcarbazole has produced one papilloma and three epitheliomata in twenty mice. Two of these epitheliomata are spindle celled (cf fig 8, Plate 20). With this compound no skin changes were noticed before 160 days (fig 3). Treatment of twenty mice with 1 2 7 8 dibenzcarbazole has produced only one epithelioma and one papilloma which arose after about 300 days and 450 days respectively. Thirty mice were treated with  $\alpha\alpha$  dinaphthylamine, thirty with  $\beta\beta$

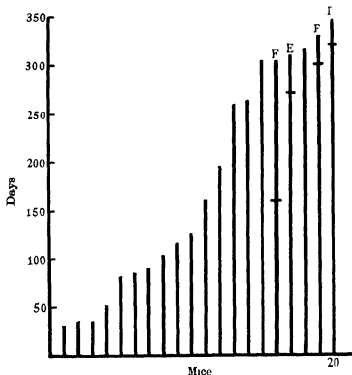


FIG 3—Mice painted with 0.3% 1 2 5 6 dibenzcarbazole

dinaphthylamine, twenty with *o*-aminoazotoluene and twenty with chrysoidine. No tumours were produced in any of these mice although in each series some mice survived over one year.

The animals which died after treatment with these compounds were examined for any neoplasms in the urinary tract. Up to the present none has been found, but it was noticed that the livers of many of the mice showed to the naked eye a nodular surface (see figs 5 and 12, Plates 19 and 21) and following this observation all livers were examined.

## CHANGES IN THE LIVER

All mice dying after treatment with 3 4 5 6 dibenzcarbazole have shown striking lesions in the liver. One animal, which died 6 days after the first painting, showed widespread fatty infiltration of hepatic cells, and after 20 days the hepatic cells in most specimens showed various degrees of degenerative change. In twelve of the seventy two livers examined, focal necroses were seen; these occurred in animals dying at all stages, from 10 to 236 days and in conjunction with the various other lesions described. In about one half of the livers it was noted that the hepatic cell nuclei were abnormally variable in size, the smaller nuclei were pyknotic, while many very large ones were seen filled with clear staining inclusions. Occasional mitoses indicated that regeneration was taking place. A synopsis of the hepatic changes is given in Table I, and these are illustrated by figs 9-17, Plates 20, 21 and 22.

The most characteristic lesion following 3 4 5 6 dibenzcarbazole painting was hyperplasia of the bile ducts. This was seen in thirty four out of forty three animals surviving for more than 30 days, and in twenty one out of twenty four of those surviving for 90 days or more. In the earliest cases (14 days) new formed bile ducts could be seen in the peri portal regions, extending along the paths of bile capillaries, appearing to elect certain lobules and to avoid others. There was a tendency for the normally flat cells to become cuboidal and even columnar. At times these new ducts were widely dilated (see figs 9 10 and 11, Plate 20). In the later stages (50 days or more) the hyperplastic ducts filled entire lobules, in these cases the organ was grossly nodular and showed numerous small white patches on section. In thirteen specimens from 55 to 236 days after the beginning of treatment the biliary growth was very disorganized. These presented a similar gross appearance to the last group, but on microscopic examination there were many areas in which the biliary cells appeared to have become disorganized and had grown in large groups with and without acini (see figs 13 and 14, Plate 21). The cells and their nuclei were considerably larger than normal so that in places it was difficult to distinguish them from nearby compressed hepatic cells. Mitoses were fairly numerous in a few of these.

In four of those livers with most marked biliary hyperplasia, all from mice dying 200 days or more after treatment was begun, there also appeared nodular areas up to 1 mm. in size of hepatic cells, with fairly numerous mitoses and containing nuclei of all sizes, but without the pyknosis or inclusions seen in earlier specimens. Many multinuclear cells could be seen

TABLE I.—SYNOPSIS OF HEPATIC LESIONS IN MICE PAINTED WITH VARIOUS SUBSTANCES

Substance	Days treated	No of mice	Papilloma or epitheloma of skin	Number with							Hepa- toma
				Focal necroses	Fatty infil- tration	Increased endo- thelium	Localized hyper- trophy	Diffuse hyper- trophy	bile duct		
3 4 5 6 Dibenzocarbazole	6-19	12	0	1	4	2	2				
	20-30	12	0	4	3	4	6				
	31-71	12	0	1	3	2	5			5	
	72-99	12	0	1	4	3	1			7	
	100-181	12	3	3	1	3	1			7	
	182-236	6	5	2	1	2	1			5	
	237-321	4	4	4	1		1			3	
1 2 5 6 Dibenzocarbazole	56-307	7	4	3	1						
1 2 7 8 Dibenzocarbazole	90-373	8	2	1	1						
o Amnosotoluene	95-104	5	0	3	1	1					
az Dinaphthylamine	180-270	4	0	2	1	1					
$\beta\beta$ Dinaphthylamine	7 240	13	0	5	5	1					
Controls (various other sub- stances see Table II)	60-720	35	4	13	7	3					



containing as many as twelve nuclei, there were a few giant cells with very large lobular nuclei, while in places rosette like structures of closely packed hepatic nuclei were seen (see figs 15 and 16, Plate 22)

One specimen after 56 days' treatment showed bands of connective tissue growing throughout all the lobules, without any change in the bile ducts. Many of the specimens with biliary hyperplasia showed slightly increased connective tissue, but in none of these was it a prominent part of the picture. In most of the livers in the series there was a considerable increase in the number of endothelial cells, and this was most striking in those animals dying during the second month, at which time biliary changes were beginning. A few scattered specimens showed perivascular infiltration with leucocytes.

None of the animals treated with other dibenzcarbazoles (1278 or 1256) or with the two dinaphthylamines have shown hypertrophic biliary or hepatic cell lesions. The various other lesions enumerated (fatty infiltration and degenerative changes in hepatic cells, increased endothelium, and perivascular leucocytosis) have been seen in these groups, focal necroses are relatively more frequent than in the group treated with the 3456 compound. Three mice treated with  $\beta\beta$  dinaphthylamine for 65-70 days showed multiple focal necroses in both liver and spleen. Four livers from mice dying after 95-104 days' painting with *o*-aminoazotoluene have been examined according to the same technique, three of these have contained focal necroses and one has shown degenerative hepatic cell changes as described above. No hypertrophic lesions were present.

If we assume that in our experiments 0.05 ml. of 0.1 or 0.3% solution has been placed on the skin at each painting, and that all of the substance is absorbed, and calculate on that basis, we find that well marked biliary hypertrophy occurs after 55 days and a total of 0.8 mg. of 3456 dibenzcarbazole, while frank hepatic cell proliferation resembling hepatoma occurs after application of 6.2 mg. of the substance. Estimating in the same way, we find that 10 mg. or more of the other dibenzcarbazoles and of the dinaphthylamines, and 4.5 mg. of *o*-aminoazotoluene have been administered without giving rise to any hypertrophic lesions of the liver.

As controls, thirty five livers were examined in mice under investigation by other workers here. The animals had died after from 1 month to 2 years of painting with various other carcinogenic and related substances, including 34 benzpyrene, methylcholanthrene, and 1256 dibenzanthracene. Table II summarizes the results found in these control mice. The majority of the control livers were essentially normal, a few showed focal necroses, fatty infiltration, or perivascular leucocytic infiltration or increased

TABLE II.—SYNOPSIS OF HEPATIC LESIONS IN CONTROL MICE PAINTED WITH VARIOUS SUBSTANCES

Substance	Days painted	No of mice	Papilloma or epu theloma of skin	Number with				
				Focal necrosis	Fatty infil tration	Increased endo thelium	Pen vascular leuco cyto sus	Increased connect- ive tissue
Methyl 3 4 benzpyrene	385	1		1				
3 4 Benzpyrene	24-155	3		1			1	
Decahydrobenzpyrene	300	2		2				1
Methylcholanthrene	120-600	4	2	2			2	
1 2 cyclo Penteno acenaphthene 3 <i>spiro</i> cyclo 2 methyl-cyclohexane*	125-720	4		1			2	
1 2 5 6 Dibenzanthracene	55-210	3	1				1	
1 2 5 6 Dibenzphenazine	50	1						
6 Methyl 3 4 benzacridine	200-330	2			1	1		
9 10 Dihydro 1 2 7 8 dibenzacridine	330	1						
9 10 Dihydro 3 4 6 7-dibenzacridine	60-330	5		3	2	1	1	
10-Phenyl 9 10 dihydro 3 4 5 6 dibenzacridine	165-330	3			1		1	1
10-Piperonyl 9 10-dihydro 3 4 5 6 dibenzacridine	130-135	2			1			
(Fed) 6 Methyl 3 4 benzacridine	100-400	3		2	1	1		
(Fed) 9 10 Dihydro 3 4 6 7-dibenzacridine	380	1		1	1			
Totals		35	3	13	7	3	8	2

\* Cook Haslewood and Robinson (1935)

endothelium None of these abnormalities were confined to any particular group of substances, and diffuse degenerative hepatic cell changes were entirely absent

We have failed to find any metastatic lesions of hepatic origin in any of our mice The biliary growth takes place by extension through lobules, the hepatic cell hypertrophy by the formation of nodular growths which press on the surrounding area

#### SARCOMA PRODUCED IN RATS WITH 3 4 5 6-DIBENZCARBAZOLE

A colloidal suspension of 3 4 5 6 dibenzcarbazole was made in the same way as that used for 1 2 5 6 dibenzanthracene (Boyland 1932) The colloidal solution contained 0.05% of the dibenzcarbazole Ten rats were injected *sub cutem* twice weekly with 2 ml of this colloidal solution One rat died after 28 days and another after 80 days with no obvious lesions

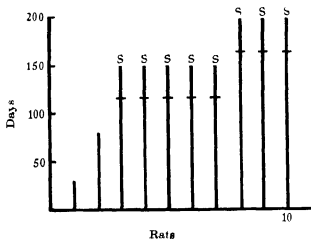


FIG 4—Rats injected *sub cutem* with 3 4 5 6 dibenzcarbazole in colloidal suspension  
S = Spindle celled tumour

After 116 days six of the rats were found to have nodules at the site of injection After 164 days' treatment five of the rats which had large tumours were killed The tumours were all spindle celled One of them was grafted into ten rats and grew in three of these One of the tumours was grafted into a second generation and one out of ten grew and has reached the fifth generation The production of tumours in these rats is shown in fig 4 and a microphotograph of the tumour which was grafted into other rats is shown in fig 18, Plate 22 All the sarcomata in rats were produced within 196 days, the liver and other organs were not markedly abnormal

## DISCUSSION

Of the compounds which have been tested 3 4 5 6 dibenzcarbazole is undoubtedly the most active. It differs from carcinogenic hydrocarbons in being slightly soluble in water and in being much more toxic to mice. The greater activity of 3 4 5 6 dibenzcarbazole as compared with 1 2 5 6 dibenzcarbazole is closely parallel to the greater activity of 3 4 5 6 dibenzacridine as compared with 1 2 5 6 dibenzacridine found by Barry and others (1935). It is noteworthy that 1 2 7 8 dibenzcarbazole is much less active. While this work has been in progress Schurch and Winterstein (1935) have reported that 1 2 benzcarbazole is possibly carcinogenic but it is evidently much less active than the 3 4 5 6 dibenzcarbazole.

Nishiyama (1935) reported hypertrophic changes in the mouse liver and Sasaki and Yoshida (1935) have observed similar lesions in rats after feeding with a diet containing *o* aminoazotoluene. It seems clear from their report that the sequence and appearance of the lesions in their experiments on mice were in some ways similar to what we have observed with 3 4 5 6 dibenzcarbazole. Both the dosage and time interval have however been very much less with the latter substance. For example we have found well marked bile duct hypertrophy in the mouse after administering an estimated dosage of 0.8 mg. of 3 4 5 6 dibenzcarbazole to the skin while according to Nishiyama at least 27 mg. of *o* aminoazotoluene must be given by mouth for the same effect. Similar comparative doses required for the development of nodular hepatic cell proliferation of the hepatoma type would be 6.2 and 350 mg. respectively. While the primary proliferation produced in mice by 3 4 5 6 dibenzcarbazole is in the bile duct cells the main change induced by *o* aminoazotoluene as described by Sasaki and Yoshida (1935) is in the hepatic cells. Strong and Smith (1936) have described the occurrence of spontaneous benign hepatoma in the CBA pure strain of mice. 3 4 5 6 Dibenzcarbazole differs from other known carcinogenic agents in producing hepatoma in addition to epitheliomata and sarcomata. An epithelioma and hepatoma can be produced in the same animal.

We wish to express our thanks to Mrs Kennaway for her careful post mortem examinations of all the experimental mice described in this paper. It was due entirely to her observations that the presence of hepatic lesions in this series of mice was first discovered. We are indebted to Dr Max Hofer and Dr A. Winterstein of Hoffmann La Roche Chemical Works Ltd. of Basle for preparing the dibenzcarbazoles used in these experiments. We are indebted to the British Empire Cancer Campaign for a grant which has assisted this investigation.

## SUMMARY

$\alpha\alpha$ - and  $\beta\beta$ -Dinaphthylamines have shown no carcinogenic activity when tested in the usual way by painting on mice. 3:4:5:6-Dibenzcarbazole and 1:2:5:6-dibenzcarbazole produced malignant changes in the skin of mice in 180 and 250 days respectively. 1:2:7:8-Dibenzcarbazole produced only one papilloma and one epithelioma in twenty mice after 500 days' treatment.

Among the mice painted with 3:4:5:6-dibenzcarbazole, 80% of those which survived over a month have shown hypertrophic biliary changes, ranging from a simple localized increase in the number of ducts in the portal areas, to diffuse growth of metaplastic biliary tissue throughout the lobules. Most of the mice surviving over 200 days have shown nodules of altered hepatic cells resembling hepatoma. It is estimated that these changes can occur after the administration of about 0.8 mg. (for bile duct hypertrophy) and 6 mg. (for hepatoma) of the substance. No metastases from the liver lesions have been seen.

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## DESCRIPTION OF PLATES

## Plate 19

FIGS. 5, 6, 7—3:4:5:6-Dibenzcarbazole. Mouse painted for 236 days.

FIG. 5—Mouse showing epithelioma and nodular liver.

FIG. 6—Epithelioma.  $\times 110$ .

FIG. 7—Metastasis of epithelioma in left axilla.  $\times 95$ . This animal showed marked biliary and hepatic cell hypertrophy.



FIG. 5



FIG. 6

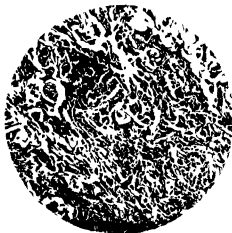


FIG. 7



Fig. 8

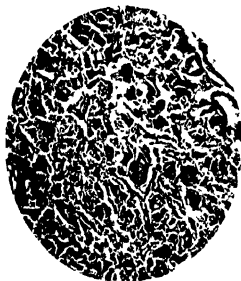


Fig. 9

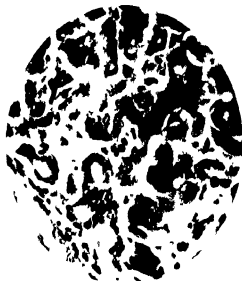
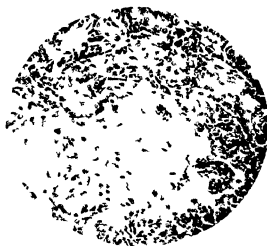


Fig. 10



Fig. 11







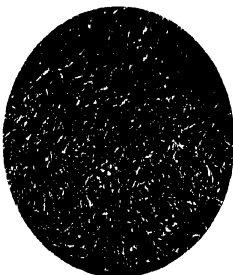
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**Plate 20**

- FIG 8**—1:2 5 6 Dibenzocarbazole Mouse painted for 307 days Spindle celled epithelioma at site of painting The liver showed only slight fatty infiltration and perivascular accumulation of leucocytes  $\times 400$
- FIG 9**—3 4 5 6 Dibenzocarbazole Liver of mouse painted for 46 days Increased bile duct growth and capillary endothelium throughout the lobule and great variation in size of hepatic cell nuclei In one place are dilated biliary ducts with cuboidal epithelium  $\times 110$
- FIG 10**—3 4 5 6 Dibenzocarbazole Mouse painted for 62 days Early localized biliary hypertrophy  $\times 370$
- FIG 11**—3 4 5 6 Dibenzocarbazole Mouse painted for 90 days Localized biliary growth, becoming more widespread than in fig 10  $\times 85$

**Plate 21**

- FIG 12**—3 4 5 6 Dibenzocarbazole Mouse painted for 121 days showing nodular appearance of liver
- FIGS 13, 14**—3 4 5 6 Dibenzocarbazole Mouse painted for 227 days Late stage of biliary growth **FIG 13**—Low power view of region showing many cells of biliary origin  $\times 110$  **FIG 14**—Detail of another region showing duct formation and mitosis (arrow)  $\times 370$

**Plate 22**

- FIGS 15, 16**—3 4 5 6 Dibenzocarbazole Mouse painted for 234 days **FIG 15**—Border of hepatoma nodule, showing compression of surrounding liver, which also shows biliary changes  $\times 110$  **FIG 16**—Detail from centre of the nodule  $\times 360$
- FIG 17**—3 4 5 6 Dibenzocarbazole Mouse painted with 0.1% solution for 321 days Border of area of bile duct hypertrophy  $\times 52$
- FIG 18**—Rat injected with 3 4 5 6 dibenzocarbazole Day 150 Spindle celled sarcoma  $\times 125$
-

# The Influence of Various Polycyclic Hydrocarbons on the Growth Rate of Transplantable Tumours

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[Plates 23, 24]

## I—INTRODUCTORY

As a result of considerations which will be discussed to some extent in the present paper, experiments were carried out to test the effect of certain of the carcinogenic hydrocarbons, studied at the Research Institute of the Royal Cancer Hospital (Cook and others 1932, Cook 1932, Barry and others 1935) on the growth rate of transplantable animal tumours. The work was also extended to include related non-carcinogenic hydrocarbons and other substances. A short preliminary account has already been published (Haddow 1935).

## II—EXPERIMENTAL

1—*Animals*—The majority of the animals used were hooded rats of the Lister Institute strain, since previous experience had shown these to be particularly suitable for use with the Jensen and Walker tumours. Wistar rats from the Glaxo laboratories proved susceptible to the Walker tumour and were also used on a few occasions. Batches were selected in which the animals were in perfect condition and of reasonable uniformity of weight (usually 100–150 g).

2—*Tumour strains and Technique of Implantation*—The rat tumours studied were the Jensen sarcoma and Walker carcinoma (strains supplied by the Imperial Cancer Research Fund), a sarcoma (LR-10) induced in the Research Institute of the Royal Cancer Hospital by the injection of 1,2,5,6-dibenzanthracene in lard (Burrows, Hieger and Kennaway 1932).



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and subsequently maintained by transplantation and a rat sarcoma induced by 3·4 benzpyrene for which the writer is indebted to Dr A M Hain of the Institute of Genetics University of Edinburgh. A few experiments were also carried out with the Rous fowl sarcoma.

Rats were prepared for tumour implantation by the epilation of an area of skin on the right flank. Next day an active tumour of 10–14 days was exposed aseptically. Thin slices were removed from the healthy periphery placed in sterile saline and divided into uniform cubes about 2–3 mm in length. Each rat of the fresh batch was then lightly anaesthetized with ether and after sterilization of the skin a paracentesis cannula containing a fragment of tumour was introduced obliquely through a small incision which was then closed with a Michel clip.

3—*Administration of Compounds*—In the early experiments the hydrocarbons were given in aqueous colloidal suspensions (0·03%) prepared as described by Boyland (1932) or for higher concentrations by the use of sodium dispersol (supplied by Imperial Chemical Industries Ltd). These preparations were sterilized by boiling or steaming. Intraperitoneal injection was employed throughout partly to secure adequate absorption and also to exclude all possibility of action by direct contact with the subcutaneous tumour. Colloidal preparations were usually given daily but later it was found much more convenient and at least equally satisfactory to give the compounds in one or two doses dissolved in sesame oil. For this purpose concentrations of 0·5 or 1·0% were prepared in the hot air oven at 100° C treatment which served at once to dissolve and to sterilize. One to five c.c. of solution was injected through a small epilated and sterilized area on the abdominal wall.

4—*General Design of Experiments*—In preliminary series individual tumours served as their own controls the rate of growth being measured daily for some time before and after administration of the substance to be tested. Later the growth of a number of treated tumours was compared with that of tumours in control animals by comparing either the daily measurements or the weights of the tumours after removal at the end of each experiment. In all cases the tumours in a given test were implants from a single tumour of the previous generation and control and experimental series were comparable in such details as age and weight. Control rats throughout received similar injections of the appropriate material e.g. the gelatin base used in the preparation of colloid or sterile sesame oil and hence were subjected to the same degree of handling and other interference as the corresponding experimental rats.

The majority of the later<sup>4</sup> experiments conformed to a common plan. Injections, usually two, were given shortly after tumour implantation. In Exps 7, 11, 12, 14, 16, 17, 18, 19, 20, 25, 27, 29, 30, 31, 32, 33 and 34 these injections were given within a period of not more than 3 days following the graft. After an average period of 3 weeks the rats were killed and their tumours removed. A statistical comparison—rigorous if necessary—was made of the weights of tumours from the control and experimental series respectively. This arrangement was preferred in most cases, since the first purpose of the present work was to test a sufficient number of selected compounds under approximately standard conditions. Nevertheless, some experiments were performed in which the time of administration of the compound—in relation to tumour implantation—differed from the above.

The procedure usually included blood counts in representative animals at the end of the experiment and the removal of tissues (usually liver, kidney, spleen, suprarenal, thyroid, pituitary, bone marrow, testis or ovary and the tumour) for histological examination.

#### A—Carcinogenic Hydrocarbons

*Exp 1—Jensen Sarcoma.* 1 2 5 6-Dibenzanthracene—Eight rats bearing healthy 10-day-old tumours were divided into two equal groups, of which one received daily 1 c.c. of a 0.03% aqueous colloidal suspension of 1 2 5 6-dibenzanthracene in 0.5% gelatin while the controls were given similar injections of the gelatin solution alone. The total dosage of 1 2 5 6-dibenzanthracene was approximately 8 mg. The growing tumours were measured daily with calipers and the product of the length, breadth and thickness in cm. taken as an index of tumour size, the result of the experiment being expressed in these terms (Table I and fig. 1). 1 2 5 6-Dibenzanthracene retarded the growth-rate considerably. That the result shown in fig. 1 is characteristic can be seen from the individual data for this experiment given in the Appendix.

TABLE I

Group	Index of mean tumour size		% increase
	1st day	11th day	
Controls (4)	4.1	37.8	822
Treated (4)	3.9	19.1	490

*Exp 2—Jensen Sarcoma.* 1 2 5 6-Dibenzanthracene—Ten rats were given four consecutive daily injections of 1 c.c. 0.03% colloidal 1 2 5 6-dibenzanthracene in gelatin while a control group of nine rats received the same

number of injections of gelatin alone. Both groups were then implanted with Jensen sarcoma from a single donor and the daily injections continued. A week later the tumours in both groups had taken successfully but those of the controls were growing faster. Daily tumour measurements were made from the 10th day after transplantation (fig. 2, which confirms the result of Exp. 1). The rats were killed and their tumours removed on the 21st day after transplantation. Table II shows the volumes of the individual tumours as determined by displacement.

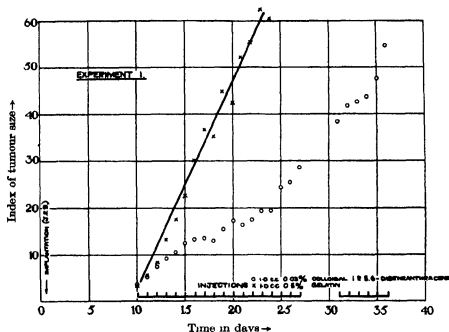


FIG. 1.—The growth of two representative tumours (control and treated) from Exp. 1. Index of tumour size = product of length, breadth and thickness.

TABLE II

Group	Treatment	Tumour volumes (c.c.) after 21 days			
		Individual			Mean
I (7 rats)	1 c.c. colloidal 12.5% 6-dibenz- anthracene daily	14	10	8	6.8
		7	5	3	
		1			
II (9 rats)	Control. 1 c.c. 0.05% gelatin daily	42	40	39	33.2
		33	33	32	
		31	25	24	

The course of the subsequent experiments will now be described in a more abbreviated form, only those details being set down which cannot well be recorded in the tables.

*Exp 3—Walker Carcinoma 1256-Dibenzanthracene*—Ten days after implantation group I received two intraperitoneal injections of 25 mg

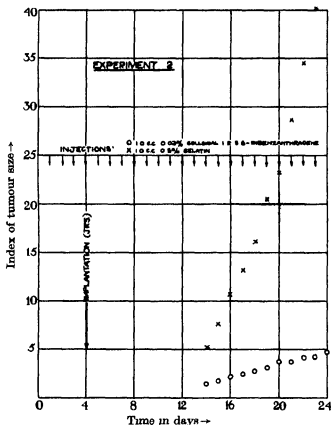


FIG. 2.—Mean tumour size (length  $\times$  breadth in cm) of control and treated tumours from Exp 2

1256-dibenzanthracene in sesame oil and group II similar injections of sesame oil alone, while group III was kept as an un.injected control. Daily measurements were made from the 5th day after implantation onward (fig. 3 and Table III for complete data see Appendix).

The experiment was continued to the 57th day, by which time all but two of the control animals had died with extremely large tumours. Meantime the treated tumours were quiescent or increasing very slowly.

TABLE III

Group	Treatment on 10th day	Index of mean tumour size		% increase
		10th day	24th day	
I (6 rats)	2 x 25 mg 1.2 5 6-dibenzanthracene	36.0	36.1	0.28
II (6 rats)	2 x 5 c.c. sesame oil	40.4	63.8	57.90
III (4 rats)	Nil	38.4	64.2	67.10

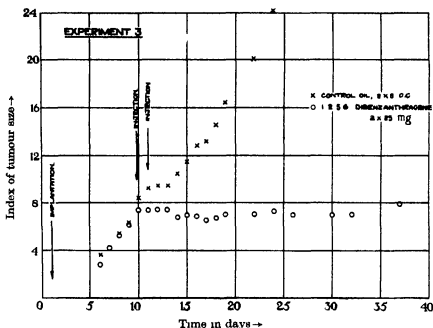


FIG. 3—Mean tumour size (length x breadth in cm) of control and treated tumours from Exp. 3

*Exp. 4—Jensen Sarcoma 1.2 5 6-Dibenzanthracene*—Two groups of eleven were injected daily with 2.5 c.c. 0.03% colloidal 1.2 5 6-dibenzanthracene in 0.5% gelatin in one case, and in the other with the same quantity of gelatin treated exactly as in the preparation of colloid except for the addition of hydrocarbon. Three rats were kept as untreated controls.

A week later, although all the grafts had taken successfully, the tumours in the rats receiving 1.2 5 6-dibenzanthracene were growing at a slower rate. This difference became increasingly obvious and the experiment was



terminated on the 21st day following implantation when the treated animals had each received 16 mg. The tumours were then removed and weighed (Table IV).

TABLE IV

Group	Treatment	Tumour weights (g) after 21 days				Mean
		Individual				
I (11 rats)	2.5 c.c colloidal 1.25 6-dibenzanthracene daily (total 16 mg.)	9.0	9.0	8.3	5.3	4.7
		4.1	3.4	3.4	3.2	
		2.7	1.9	1.7		
II (11 rats)	2.5 c.c 0.5% gelatin	30.0	27.6	26.0	21.5	17.1
		20.8	19.1	14.5	9.4	
		8.7	7.1	3.4		
III (3 rats)	Nil	28.0	25.5	14.5		22.7

*Exp. 5—Jensen Sarcoma 1.25 6-Dibenzanthracene*—Group I received daily from the day of grafting 5.0 c.c. colloidal 1.25 6-dibenzanthracene, and group II the same of gelatin which had been treated with acetone as in the preparation of colloid. The tumours were removed on the 18th day, when each rat in group I had received about 30 mg. dibenzanthracene (Table V, rats which proved resistant to the graft are omitted)

TABLE V

Group	Treatment	Tumour weights (g) after 18 days				
		Individual				Mean
I (8 rats)	5 c.c. colloidal 1.25 6-dibenzanthracene daily (total 30 mg.)	3.4	2.4	2.2	1.7	1.6
		1.5	0.9	0.8	0.4	
II (5 rats)	5 c.c. 0.5% gelatin daily	20.0	15.5	10.2		12.0
		7.7	7.0			

*Exp. 6—Jensen Sarcoma 1.25 6-Dibenzanthracene*—Two groups of twelve were used and treatment began on the day of grafting. Tumours were removed after 14 days, when the experimental rats had received about 12 mg (Table VI)

TABLE VI

Group	Treatment	Tumour weights (g.) after 14 days				
		Individual				Mean
I (9 rats)	2.5 c.c. colloidal 1.25 6-dibenzanthracene daily (total 12 mg.)	4.0	2.0	2.0	2.0	1.7
		1.5	1.5	1.0	0.5	
		0.5				
II (10 rats)	2.5 c.c. 0.5% gelatin daily	10.0	10.0	7.0	6.0	5.8
		6.0	6.0	5.0	4.0	
		2.0	2.0			

*Exp. 7—Walker Carcinoma. 1:2:5:6-Dibenzanthracene*—Thirty rats were grafted and fifteen of them then received two doses of 5.0 c.c. of a 0.5% solution of 1:2:5:6-dibenzanthracene in sesame oil on successive days, while the remainder were given the same quantity of oil alone. Table VII and fig. 4 show the end-result, which represents the most intense inhibitory effect so far obtained in these experiments.

TABLE VII

Group	Treatment	Tumour weights (g.) after 21 days				
		Individual				Mean
I (15 rats)	2 × 25 mg. 1:2:5:6-dibenzanthracene in oil	2.2	1.4	1.3	1.3	1.05
		1.1	1.1	1.0	1.0	
		1.0	0.9	0.9	0.7	
		0.7	0.6	0.6		
II (15 rats)	2 × 5 c.c. sesame oil	49.6	46.6	38.0	37.5	30.90
		35.7	33.2	29.7	29.7	
		29.2	29.0	28.5	22.9	
		20.5	17.4	15.7		

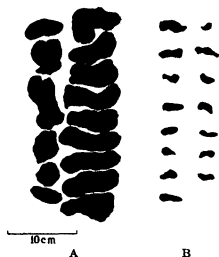


FIG. 4—Exp. 7; Walker carcinoma, 21 days. A, tumours from control rats mean weight 30.9 g. B, tumours from rats treated with 50 mg. 1:2:5:6-dibenzanthracene. mean weight 1.05 g.

*Exp. 8—Rous Fowl Sarcoma 1:2:5:6-Dibenzanthracene*—Twenty-four Brown Leghorn fowls of about 500 g. were grafted in the breast muscle. Half of them were then given three doses of 30 mg. 1:2:5:6-dibenzanthracene in oil on successive days by intramuscular injection on the opposite

side while the rest received sesame oil alone. There was considerable mortality in both groups. The survivors were killed on the 18th day and their tumour tissue dissected out and weighed. A significant inhibition was produced, similar in degree to that obtained in the foregoing experiments with rat tumours (Table VIII).

TABLE VIII

Group	Treatment	Weight of tumour tissue (g) after 18 days					Mean
		Individual					
I (7 fowls)	3 x 30 mg 1 2 5 6-dibenz- anthracene in oil	14 2	11 2	10	4	3	6.6
II (9 fowls)	Sesame oil alone	53 28	46 22	46 14	42 13	35	33.2

*Exp 9—Jensen Sarcoma 5 6-Cyclopenteno-1.2-benzanthracene*—Forty rats were grafted. Twenty then received daily 0.03% colloidal 5.6-cyclopenteno-1.2-benzanthracene in 0.5% gelatin, ten received the control solution of gelatin alone and ten remained untreated. Of group I, one died and two proved resistant to the graft. The rest were killed on the 18th day, when those in the first group had each received 15 mg. (Table IX and fig. 5).

TABLE IX

Group	Treatment	Tumour weights (g) after 18 days					
		Individual					Mean
I (17 rats)	25 c.c. colloidal 5.6-cyclo- penteno-1 2-benzanthra- cene daily (total 15 mg)	8.1	7.9	6.4	4.1	4.1	3.5
		4.0	3.4	3.3	2.8	2.5	
		2.4	2.3	2.3	2.0	1.7	
		1.6	0.4				
II (10 rats)	25 c.c. 0.5% gelatin daily	33.4	29.7	29.5	24.8	15.9	18.2
		15.9	13.2	8.9	6.4	4.7	
III (10 rats)	Nil	49.1	40.4	34.6	34.2	21.0	24.9
		14.7	14.6	14.6	13.4	12.5	

Here one could compare the controls subjected to daily injection, etc. with those not so handled, as regards the mean weight of the tumours in each group. Although the mean tumour weight was higher in the latter group, the application of the *t* method (Fisher 1932) showed that the difference was not significant, the value of *P* being 0.2–0.3. On the other hand the difference between the means of groups I and II in Table IX was highly significant ( $P < 0.01$ ), as was the case for all the experiments so far described (see also Table XXXVI).

*Exp 10—Jensen Sarcoma. Sodium-1 2:5:6-dibenzanthracene-9 10-endo- $\alpha$ - $\beta$ -succinate*—This compound was tested in view of its special interest as a water-soluble carcinogenic compound (Burrows and Cook 1936).

Twenty-four rats were grafted and divided into two equal groups. One received daily the substance in partial solution in sterile water while the other received the solvent only. On the 18th day the treated rats had received about 100 mg of succinate (Table X). At autopsy, the treatment appeared to have produced some irritation of the peritoneum, an effect not observed with the compounds studied thus far.

TABLE X

Group	Treatment	Tumour weights (g) after 18 days				
		Individual				Mean
I (12 rats)	Na-1 2 5 6-dibenzanthracene-9 10-endo- $\alpha$ - $\beta$ -succinate, c 100 mg.	9.5	7.2	7.1	6.5	5.2
		6.3	5.3	4.7	3.9	
		3.9	3.7	2.9	1.8	
II (12 rats)	Control	36.4	31.8	29.3	27.2	18.7
		17.9	17.2	17.1	14.6	
		10.0	8.6	8.3	6.4	



FIG. 5



FIG. 6

FIG. 5—Exp 9; Jensen sarcoma, 18 days. A, tumours from injected control rats: mean weight 18.2 g. B, tumours from un.injected control rats: mean weight 24.9 g. C, tumours from rats treated with 15 mg 5:6-cyclopenteno-1 2-benzanthracene: mean weight 3.5 g.

FIG. 6—Exp 11, Walker carcinoma, 28 days. A, tumours from control rats: mean weight 31.0 g. B, tumours from rats treated with 50 mg 3:4-benzpyrene: mean weight 2.1 g.

*Exp. 11—Walker Carcinoma. 3 4-Benzpyrene\**—Twenty-five rats were grafted. Group I were then given two injections of 25 mg. 3 4-benzpyrene in sesame oil on successive days, while the rest received oil alone. Three treated animals died during the period of 28 days (Table XI and fig. 6).

TABLE XI

Group	Treatment	Tumour weights (g.) after 28 days					Mean
		Individual					
I	2 x 25 mg. 3 4-benzpyrene	58	41	26	21	1.7	21
(9 rats)	in oil	11	07	06	02		
II	2 x 5 c.c. sesame oil	59.2	48.1	46.1	37.4	35.0	31.0
(13 rats)		33.1	31.3	29.5	27.6	26.4	
		20.0	6.4	3.2			

*Exp. 12—Walker Carcinoma 3 4 5 6-Dibenzacridine*—This compound was chosen as being related to the polycyclic hydrocarbons and possessing moderate carcinogenicity, the effects of which are delayed as compared with say 1 2 5 6-dibenzanthracene (Barry and others 1935).

Twenty rats were grafted in two groups of fifteen and five. These were injected with 50 mg. 3 4 5 6-dibenzacridine in oil and oil alone respectively. The mean weights after 32 days (Table XII) were examined statistically and the value for *P* found to be less than 0.01. The inhibition is thus of considerable significance although much less than in the case of the substances used in previous experiments

TABLE XII

Group	Treatment	Tumour weights (g.) after 32 days			
		Individual			Mean
I (13 rats)	2 x 25 mg. 3 4 5 6-dibenz- acridine	45.5	36.7	36.4	25.3
		32.2	29.2	29.1	
		27.5	25.5	25.2	
		12.0	11.5	10.7	
		7.5			
II (5 rats)	2 x 5 c.c. sesame oil	79.5	66.2	46.2	52.5
		41.4	29.0		

#### B—Chrysene and Certain Compounds of Benzanthracene Type

The following experiments were carried out with synthetic chrysene and certain compounds of benzanthracene type, the carcinogenicity of which is weak, doubtful, or in some cases (e.g. 3-methyl- and 7-methyl-1,2-benzanthracene), nil

\* Originally termed 1,2-benzpyrene. See Cook and others (1936, p. 2)

*Exp. 13—Jensen Sarcoma. Chrysene*—Fifteen rats were grafted. One group received colloidal chrysene daily in 0.75% gelatin and the other gelatin solution alone. At the end of the experiment the mean weight of treated tumours was less than one-third that of the controls (Table XIII).

TABLE XIII

Group	Treatment	Tumour weights (g) after 17 days				
		Individual				Mean
I (8 rats)	Colloidal chrysene (0.03% in 0.75% gelatin daily) total 30 mg.	5.0	4.1	4.0	3.6	2.54
		1.2	1.2	0.7	0.5	
II (7 rats)	0.75% gelatin daily	18.0	14.3	11.6	4.6	8.20
		3.6	3.1	2.3		

*Exp. 14—Walker Carcinoma. Chrysene*—Thirty rats after grafting were divided into two groups which received chrysene in oil and oil alone respectively, in two doses on successive days. Again chrysene produced considerable inhibition of the tumour growth rate (Table XIV).

TABLE XIV

Group	Treatment	Tumour weights (g) after 19 days				
		Individual				Mean
I (12 rats)	2 x 30 mg. chrysene in oil	6.0	5.6	4.6	3.6	2.34
		2.0	1.4	1.2	1.0	
		0.9	0.7	0.6	0.5	
II (13 rats)	2 x 5 c.c. sesame oil	34.7	32.2	28.0	27.5	22.60
		26.2	26.1	23.8	23.8	
		21.4	21.3	15.2	9.4	
		4.7				

*Exp. 15—Jensen Sarcoma. 1,2-Benzanthracene*—1,2-benzanthracene was tested simultaneously with 5,6-cyclopenteno-1,2-benzanthracene (see Exp. 9). Of thirty rats grafted, twenty received colloidal 1,2-benzanthracene daily—to a total dosage of 15 mg.—while ten received the control solution alone. The treatment resulted in a moderate degree of inhibition (Table XV and fig. 7).

TABLE XV

Group	Treatment	Tumour weights (g) after 18 days					
		Individual					Mean
I (20 rats)	2.5 c.c. 1,2-benzanthracene daily (total 15 mg.)	21.2	17.6	17.0	14.7	11.8	7.5
		10.6	9.6	9.2	6.1	5.3	
		4.7	4.1	3.6	3.4	3.3	
		3.0	2.1	1.5	1.1	1.0	
II (10 rats)	2.5 c.c. 0.5% gelatin daily	33.4	29.7	29.5	24.8	15.9	18.2
		15.9	13.2	8.9	6.4	4.7	



FIG 7—Exp 15, Jensen sarcoma, 18 days A, tumours from injected control rats: mean weight 18.2 g B, tumours from uninjected control rats mean weight 24.9 g. C, tumours from rats treated with 15 mg 1.2-benzanthracene mean weight 7.5 g

*Exp 16—Walker Carcinoma 1.2-Benzanthracene*—Twelve of twenty-four rats were given 1.2-benzanthracene in sesame oil and the rest oil alone, in two daily doses immediately after implantation (Table XVI)

TABLE XVI

Group	Treatment	Tumour weights (g) after 21 days				
		Individual				Mean
I	2 x 30 mg 1 2-benzanthracene in oil	5.3	5.0	4.0	2.2	2.6
(8 rats)		1.6	1.4	0.7	0.6	
II	2 x 5 c.c. sesame oil	25.4	22.5	21.1	20.4	16.7
(12 rats)		18.6	17.0	16.2	15.7	
		14.6	13.0	11.1	5.0	

*Exp 17—Walker Carcinoma. 3-Methyl-1.2-benzanthracene*—3-Methyl-1.2-benzanthracene was given to fifteen rats in two doses on the days immediately after implantation. The substance was tested simultaneously with 3.4.5.6-dibenzacridine (Exp 12), with common controls. Thirteen treated animals bore tumours at the end of the experiment (Table XVII)

*Exp 18—Walker Carcinoma 4-Methyl-1.2-benzanthracene. 7-Methyl-1.2-benzanthracene*—On the 2 days following implantation, three groups of fifteen rats were injected with 4-methyl-1.2-benzanthracene, 7-methyl-1.2-benzanthracene and control oil respectively (Table XVIII).

TABLE XVII

Group	Treatment	Tumour weights (g.) after 32 days			
		Individual			Mean
I (13 rats)	2 x 25 mg. 3-methyl-1 2-benzanthracene in oil	14.5	14.5	11.1	7.7
		8.8	7.1	6.5	
		5.7	5.1	4.0	
		2.7		3.0	
II (5 rats)	2 x 5 c c sesame oil	79.5	66.2	46.2	52.5
		29.0		41.4	

TABLE XVIII

Group	Treatment	Tumour weights (g) after 15 days			
		Individual			Mean
I (10 rats)	2 x 25 mg. 4-methyl-1 2-benzanthracene in oil	3.3	2.1	1.2	1.3
		1.0	1.0	1.0	
		0.9	0.7	0.9	
II (12 rats)	2 x 25 mg 7-methyl-1 2-benzanthracene in oil	3.0	2.8	2.5	1.4
		1.1	1.1	1.1	
		0.9	0.7	0.7	
III (14 rats)	2 x 5 c c sesame oil	16.2	12.7	10.5	8.5
		8.6	8.3	8.1	
		7.2	7.0	5.0	
		4.6		4.6	

*Exp. 19—Walker Carcinoma 6-Methyl-1 2-benzanthracene*—Two groups of twelve implanted rats were given 6-methyl-1 2-benzanthracene in oil, and oil alone, respectively (Table XIX).

TABLE XIX

Group	Treatment	Tumour weights (g) after 30 days			
		Individual			Mean
I (10 rats)	2 x 25 mg 6-methyl-1 2-benzanthracene in oil	5.3	4.5	4.3	3.0
		3.4	2.6	2.2	
		1.4	0.5	1.6	
II (8 rats)	2 x 5 c c sesame oil	52.9	43.4	33.4	32.9
		28.7	28.7	26.8	

### C—Related Oestrogenic Compounds

In a study of the oestrogenic activity of condensed-ring compounds, Cook and others (1934) described work carried out with oxygen derivatives of phenanthrene and certain diols related to 1·2 5,6-dibenzanthracene. The



most active oestrogens in these classes were 1-keto-1:2:3:4-tetrahydro-phenanthrene and the di-*n*-propyl compound respectively. The following experiment was performed to determine the action of these substances on the growth of the Walker tumour.

*Exp 20—Walker Carcinoma 1-Keto-1 2 3 4-tetrahydrophenanthrene. 9 10-Dihydroxy-9 10-di-n-propyl-9 10-dihydro-1 2 5 6-dibenzanthracene—* Forty-five rats were implanted and used in groups of fifteen to test these substances with a common control. The phenanthrene derivative proved quite inactive as regards growth-inhibiting power while the dibenzanthracene diol showed moderate potency (Table XX and fig 8)

TABLE XX

Group	Treatment	Tumour weights (g) after 28 days				
		Individual				Mean
I (15 rats)	2 x 25 mg 1 keto-1 2 3 4-tetrahydrophenanthrene	45.1	42.2	35.5	35.0	29.5
		34.8	34.2	33.5	31.8	
		30.9	29.6	26.9	25.4	
		14.8	13.7	9.8		
II (13 rats)	2 x 25 mg 9 10-dihydroxy-9 10-di-n-propyl-9 10-dihydro-1 2 5 6-dibenzanthracene	9.7	6.7	6.5	6.4	4.2
		6.0	3.9	3.8	2.7	
		2.7	2.7	2.1	0.9	
		0.9				
III (15 rats)	2 x 5 c.c. sesame oil	54.7	38.4	37.1	28.4	23.6
		26.8	23.9	21.6	21.6	
		20.1	19.5	15.1	14.6	
		13.9	9.7	8.8		

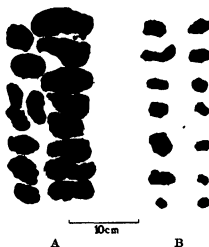


FIG. 8—Exp 20, Walker carcinoma, 28 days A, tumours from control rats mean weight 23.6 g B, tumours from rats treated with 50 mg 9 10-dihydroxy-9 10-di-n-propyl-9 10-dihydro-1 2 5 6-dibenzanthracene, mean weight 4.2 g.

Group	Treatment	Tumour weights (g) after 15 days						
		Individual					Mean	
I (11 rats)	2.5 cc colloidal phenanthrene daily	21	19	16	16	12	10	11.5
		9	7	6	6	5		
II (6 rats)	2.5 cc. 0.5% gelatin daily	19	11	10	9	9	9	11.2

*Exp. 24—Jensen Sarcoma. 1:2-Cyclopentenophenanthrene*—This test was made concurrently with Exp 13, the controls being common to both. The treated animals were eight in number and each received a total of 30 mg. in the form of colloid. Far from there being any inhibition, the mean weight of the treated tumours was greater than that of the controls (Table XXIV). However, this difference proved not to be significant, the value of *P* lying between 0.2 and 0.3.

TABLE XXIV

Group	Treatment	Tumour weights (g ) after 17 days				
		Individual				Mean
I (8 rats)	Colloidal 1:2-cyclopentenophenanthrene 0.03 % in 0.75 % gelatin daily (total 30 mg )	24.6	21.2	16.6	12.2	12.5
		11.5	6.3	5.3	2.2	
II (7 rats)	0.75 % gelatin daily	18.0	14.3	11.6	4.6	8.2
		3.6	3.1	2.3		

*Exp. 25—Walker Carcinoma. 1:2-Cyclopentenophenanthrene; dehydronorcholene*—Forty-five rats were grafted and divided into three equal batches. Group I received 1:2-cyclopentenophenanthrene in two injections in oil, group II the same quantity of dehydronorcholene, while group III were given the equivalent of sesame oil (Table XXV). Although dehydronorcholene proved completely inactive, the mean weight of the tumours treated with 1:2-cyclopentenophenanthrene was considerably greater than that of the control group, as also occurred in Exp. 24. In this case the *t* method gave a value for *P* between 0.1 and 0.05.

TABLE XXV

Group	Treatment	Tumour weights (g.) after 34 days						Mean
		Individual						
I (15 rats)	2 x 25 mg. 1:2-cyclophenophenanthrene in oil	73.0	72.1	69.7	61.4	58.5		49.0
		52.2	50.6	48.5	48.2	45.5		
		44.2	43.2	40.5	16.2	11.6		
II (12 rats)	2 x 25 mg. dehydronorcholene in oil	72.0	61.7	46.4	44.5	40.0		35.8
		38.7	37.4	29.4	26.0	15.0		
		10.5	8.7	-				
III (13 rats)	2 x 5 c.c. sesame oil	72.4	68.2	51.2	48.0	47.0		36.4
		44.5	26.5	23.7	22.0	21.4		
		20.5	14.0	13.6				

*Exp. 26—Walker Carcinoma. Pyrene; fluoranthene*—Forty-five rats were used to test pyrene and fluoranthene, which were administered in

colloidal form by daily injection. The compounds had no influence of any kind on the rate of growth (Table XXVI)

TABLE XXVI

Group	Treatment	Tumour weights (g) after 21 days					
		Individual					Mean
I (14 rats)	Colloidal pyrene (total 40 mg)	29.1	24.5	21.1	20.1	17.6	16.2
		17.2	16.8	15.3	15.3	11.0	
		10.3	9.9	9.7	9.5		
II (15 rats)	Colloidal fluoranthene (total 40 mg)	29.4	27.0	23.9	23.8	18.7	17.2
		16.9	16.7	16.4	16.1	15.2	
		15.0	13.5	10.5	9.1	5.4	
III (15 rats)	Control gelatin	30.1	23.7	22.4	19.1	18.0	15.8
		17.8	17.1	16.5	15.7	13.2	
		12.0	9.9	8.5	8.0	4.7	

*Exp. 27—Walker Carcinoma Pyrene*—This test was carried out simultaneously with Exp. 7, a group of fifteen rats serving as common controls to two experimental series. The rats were implanted on the same occasion from a single donor, and the time of injection was the same for all. In this part of the experiment two doses of 25 mg pyrene in oil were given on successive days. This produced no interference with the normal rate of tumour growth, and the final result (Table XXVII and fig. 9) presents a striking contrast to that of Exp. 7 (Table VII and fig. 4).

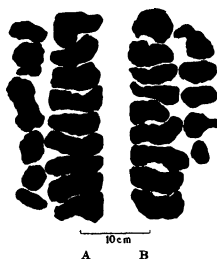


FIG. 9.—Exp. 27; Walker carcinoma, 21 days. A, tumours from control rats: mean weight 30.9 g. B, tumours from rats treated with 50 mg pyrene: mean weight 32.0 g.

TABLE XXVII

Group	Treatment	Tumour weights (g.) after 21 days					
		Individual					Mean
I (14 rats)	2 x 25 mg pyrene in oil	48.9	46.2	40.9	40.6	37.4	32.0
		37.0	36.3	33.4	27.4	25.2	
		23.5	20.3	17.9	13.5		
II (15 rats)	2 x 5 c.c. sesame oil	49.6	46.6	38.0	37.5	35.7	30.9
		33.2	29.7	29.7	29.2	29.0	
		28.5	22.9	20.5	17.4	15.7	

*Exp. 28—Rous Fowl Sarcoma. Pyrene*—Concurrently with *Exp. 8* twelve fowls implanted with the Rous sarcoma were each given 90 mg. pyrene in oil. There was undue mortality in both experimental and control groups, but pyrene had no inhibitory effect (Table XXVIII). The mean weight of the test tumours was actually considerably greater than that of the controls, but the difference was without significance ( $P = 0.2-0.3$ ).

TABLE XXVIII

Group	Treatment	Weight of tumour tissue (g.) after 18 days				
		Individual				Mean
I (7 fowls)	3 x 30 mg pyrene in oil	76 32	62 8	60 46	32	45.1
II (9 fowls)	Sesame oil alone	53 28	46 22	46 14	42 13	33.2

*Exp. 29—Walker Carcinoma. Triphenylene*—Twenty-four rats were used in two groups. The mean weight of the treated tumours at the 26th day was less than that of the controls (Table XXIX and fig. 10) but the difference was without significance ( $P$  between 0.5 and 0.4).

TABLE XXIX

Group	Treatment	Tumour weights (g.) after 26 days					
		Individual					Mean
I (10 rats)	2 x 25 mg triphenylene in oil	47.2	43.5	38.9	38.4	31.0	29.6
		26.0	25.5	21.9	13.0	9.6	
II (9 rats)	2 x 5 c.c. sesame oil	71.1	46.2	43.1	38.6	34.2	35.2
		30.5	22.4	20.4	10.0		

*Exp. 30—Walker Carcinoma. Dodecahydro-1.2-benzanthracene* (Cook and Hewett 1934). *Perylene*—Forty-six implanted rats were used to test the influence of these substances in doses of 50 mg. There was no significant

difference between the mean weights of the tumours treated with dodecahydro-1 2-benzanthracene and their controls (Table XXX) and although the mean weight of the perylene-treated tumours (fig 11) was lower than that of the controls by several grams, the calculated value for  $P$  was 0.1.



FIG 10

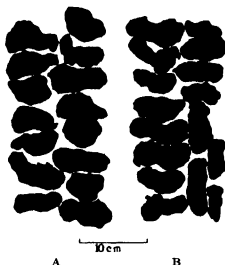


FIG 11

FIG 10—Exp 29, Walker carcinoma, 26 days. A, tumours from control rats mean weight 35.2 g. B, tumours from rats treated with 50 mg triphenylene mean weight 29.6 g

FIG 11—Exp 30, Walker carcinoma, 32 days. A, tumours from control rats mean weight 40.0 g. B, tumours from rats treated with 50 mg. perylene mean weight 33.7 g

TABLE XXX

Group	Treatment	Tumour weights (g) after 32 days			
		Individual			
I (15 rats)	2 x 25 mg dodecahydro- 1 2-benzanthracene	63.5	58.7	54.0	52.0
		51.9	44.1	41.5	39.8
		38.5	38.0	36.8	27.5
		27.5	26.9	24.2	
					41.6
II (16 rats)	2 x 25 mg. perylene in oil	56.9	52.1	50.1	47.5
		41.5	37.4	35.9	35.2
		32.3	31.9	30.9	21.7
		20.5	18.9	14.8	12.2
					33.7
III (15 rats)	2 x 5 c.c. sesame oil	47.9	47.9	43.7	42.0
		41.7	41.5	40.5	40.0
		39.8	39.6	38.6	38.1
		36.5	34.3	29.2	
					40.0

Exp. 31—*Walker Carcinoma 1.9-Benzanthrone, diphenylene oxide*—Thirty-six rats were used in three equal groups. Although the mean weight of the treated sets was lower than that of the controls (Table XXXI and fig. 12), no significance could be attached to the differences. The range of weights covered by the individual data was the same in all these groups, and the value of *P* for each half of the experiment was as high as 0.2.

TABLE XXXI

Group	Treatment	Tumour weights (g.) after 30 days			
		Individual			Mean
I (12 rats)	2 × 25 mg. 1.9-benzanthrone in oil	68.8	50.6	43.8	27.3
		36.4	34.5	21.0	
		17.0	15.0	10.7	
		10.6	10.6	8.5	
II (11 rats)	2 × 25 mg. diphenylene oxide in oil	78.3	37.6	36.2	27.3
		33.3	29.2	24.4	
		16.1	16.1	13.8	
		8.9	6.5		
III (9 rats)	2 × 5 c.c. sesame oil	69.9	46.2	45.2	38.2
		43.4	41.3	30.6	
		29.4	29.2	8.9	

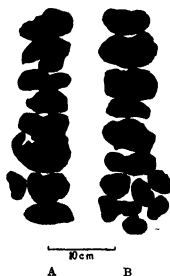


FIG. 12—Exp. 31; Walker carcinoma, 30 days. A, tumours from control rats: mean weight 38.2 g. B, tumours from rats treated with 50 mg 1.9-benzanthrone: mean weight 27.3 g.

## E—Experiments with Tumours of Known Causation

The following experiments were carried out with two transplantable rat sarcomata of known causation. The first, produced in Dr Hain's laboratory by the subcutaneous injection of 3 4-benzpyrene, appeared some 5 months after the injections and proved to be easily propagable by grafting. A number of experiments were carried out within the first few generations. The second tumour was a sarcoma induced in the Research Institute of the Royal Cancer Hospital by means of 1 2 5 6-dibenzanthracene and subsequently maintained in serial transplantation for 3 years. It was used in the present work in its 87th generation.

*Exp. 32—The Influence of 3 4-benzpyrene on the 4th Generation Transplants of a Tumour Induced by the Same Compound*—Thirty-two rats were implanted from a healthy tumour of the 3rd generation. Half of them were at once given 60 mg 3 4-benzpyrene in two doses in oil on successive days while the remainder received the control oil alone. The mean weight of the control tumours was just over twice that of the treated group (Table XXXII). This difference was significant since the value for *P* lay between 0.02 and 0.01.

TABLE XXXII

Group	Treatment	Tumour weights (g) after 24 days				
		Individual				Mean
I (14 rats)	2 × 30 mg 3 4-benzpyrene in oil	64.7	21.6	18.7	15.8	15.3
		15.3	14.3	13.3	12.7	
		12.1	10.3	8.9	3.5	
		1.9	1.4			
II (14 rats)	Control oil	68.2	57.3	56.0	52.9	33.2
		40.2	39.9	34.5	28.9	
		26.5	21.5	19.0	14.8	
		2.7	1.8			

*Exp. 33—The Influence of 1 2 5 6-dibenzanthracene on the 6th Generation Transplants of a Tumour Induced by 3 4-benzpyrene*—Thirty animals were implanted from a 5th generation sarcoma and half of them at once given 50 mg. 1 2 5 6-dibenzanthracene (Table XXXIII).

*Exp. 34—The Influence of 3-methyl-1 2-benzanthracene on the 5th Generation Transplants of a Tumour Induced by 3 4-benzpyrene*—Of twenty rats implanted from a healthy tumour of the 4th generation half were given 50 mg 3-methyl-1 2-benzanthracene in oil and the remainder oil alone (Table XXXIV).

*Exp. 35—The Influence of 1 2 5 6-dibenzanthracene on the 87th Generation Transplants of a Tumour Induced by the Same Compound*—Thirty rats



TABLE XXXIII

Group	Treatment	Tumour weights (g) after 24 days				
		Individual				Mean
I (11 rats)	2 x 25 mg 1 2 5 6 dibenz anthracene in oil	18.9	12.5	6.5	6.3	6.4
		6.0	6.0	4.7	3.1	
		3.0	2.2	1.7		
II (12 rats)	2 x 5 c.c. sesame oil	56.1	33.4	30.9	30.7	23.6
		28.7	26.6	22.0	18.2	
		14.1	11.0	6.9	4.4	

TABLE XXXIV

Group	Treatment	Tumour weights (g) after 26 days				
		Individual				Mean
I (9 rats)	2 x 25 mg 3 methyl 1 2 benzanthracene	26.4	13.5	11.6	10.7	9.7
		8.4	8.1	4.5	2.1	
		2.0				
II (8 rats)	2 x 5 c.c. sesame oil	81.8	63.0	62.2	44.7	48.4
		40.7	37.1	34.4	23.2	

were implanted from a healthy tumour of the 86th generation of the LR-10 sarcoma. Fifteen of these were then given daily injections of colloidal 1 2 5 6 dibenzanthracene for 17 days during which time each received about 30 mg of the hydrocarbon. The remaining animals were given the control material (0.5% gelatin) alone. The result was a moderate degree of inhibition (Table XXXV).

TABLE XXXV

Group	Treatment	Tumour weights (g) after 17 days					
		Individual					Mean
I (14 rats)	Colloidal 1 2 5 6 dibenzan thracene daily (total 30 mg)	3.5	3.2	2.8	2.5	2.5	1.9
		2.5	2.1	1.9	1.6	1.6	
		0.9	0.7	0.6	0.5		
II (15 rats)	Control gelatin	12.3	11.6	9.9	8.8	7.7	6.1
		7.0	6.2	6.0	4.8	4.7	
		4.1	2.6	2.4	1.8	1.5	

### III—DISCUSSION

#### A—Results

Table XXXVI summarizes the experiments already described. The ratio "mean weight of tumours from treated animals: mean weight of control tumours" is an index of the inhibiting effect, if any, produced. Throughout

the work the usual convention  $P=0.05$  was taken as expressing the limit of significance

It is obvious that compounds with acknowledged carcinogenic activity produced inhibition of the growth of the tumours used. Confirmatory results have recently been published by Morelli and Guastalla (1936) who obtained inhibition of the Jensen and Walker tumours with 3,4-benzopyrene.

Secondly, inhibitory power was shown usually to a somewhat less extent by chrysene, 1,2-benzanthracene, 4-methyl-1,2-benzanthracene, 6-methyl-1,2-benzanthracene, 3-methyl-1,2-benzanthracene and 7-methyl-1,2-benzanthracene, substances whose carcinogenicity is either exceedingly weak (as in the case of the first four named) or nil, as in the last two. Chrysene was stated by Twort and Fulton (1930) and Bottomley and Twort (1934) to possess a low order of carcinogenic power, while Barry and Cook (1934) and Barry and others (1935) found only slight activity in experiments with highly purified chrysene derived from coal tar. Later work was carried out with synthetic chrysene not open to contamination with constituents of tar (Cook and others 1936). The synthetic compound was applied to the skin of 20 mice of which 5 lived for more than 440 days. The last mouse died on the 853rd day, bearing a large epithelial tumour which had appeared first on the 711th day; this showed some downgrowth but did not reach the superficial layer of voluntary muscle. No other mouse showed any other tumour. Hence the carcinogenic power of chrysene is of an extremely low order. In addition, the subcutaneous injection in ten rats of solutions of chrysene in lard gave spindle-celled tumours in two cases. The same authors state that while 1,2-benzanthracene itself showed little if any carcinogenicity, the introduction of alkyl groups into positions 5 and 6 invariably led to activity.

Lastly, the remaining non-carcinogenic compounds proved quite inactive as regards growth-inhibiting power: the mean weight of the treated tumours was less than that of the controls in five out of fifteen experiments, and in only one of these (Exp. 30, perylene) was the value for  $P$  as low as 0.1. As has been mentioned, the case of 1,2-cyclopentenophenanthrene was of additional interest, since the data suggested that this compound might actually possess a stimulating action; the differences, however, were not judged significant. According to Cook and others (1936), 1,2-cyclopentenophenanthrene applied to the skin of mice was non-carcinogenic, although the subcutaneous injection of a solution in lard gave a spindle-celled tumour—which, however, did not grow even in autograft—in one of ten rats.

TABLE XXXVI

Exp. no	Tumour	Compound	Dose (mg)	Duration of exp (days)	Mean wt treated tumours/mean wt control tumours	No of animals	P
2	Jensen sarcoma	1 2 5 6 dibenzanthracene	8	21	0.20	16	<0.01
4	"	"	16	21	0.27	22	<0.01
5	"	"	30	18	0.13	13	<0.01
6	"	"	12	14	0.29	19	<0.01
7	Walker carcinoma	"	21	21	0.03	30	<0.01
8	Rous sarcoma	"	90	18	0.20	16	<0.01
9	Jensen sarcoma	5 6 cyclopenteno 1 2 benz anthracene	15	18	0.19	27	<0.01
10	"	Sodium 1 2 5 6-dibenzanthracene 9 10-endo $\alpha$ $\beta$ succinate	100	18	0.27	24	<0.01
11	Walker carcinoma	3 4 benzpyrene	50	28	0.07	22	<0.01
12	"	3 4 5 6-dibenzacridine	50	32	0.48	18	<0.01
13	Jensen sarcoma	Chrysene	30	17	0.30	15	0.05-0.02
14	Walker carcinoma	"	60	19	0.10	25	<0.01
15	Jensen sarcoma	1 2 benzanthracene	15	18	0.41	30	<0.01
16	Walker carcinoma	"	60	21	0.15	20	<0.01
17	"	3 methyl 1 2 benzanthracene	50	32	0.14	18	<0.01
18	"	4 methyl 1 2 benzanthracene	50	15	0.15	24	<0.01
19	"	7 methyl 1 2 benzanthracene	50	15	0.16	26	<0.01
18	"	6 methyl 1 2 benzanthracene	50	30	0.09	18	<0.01
20	"	1 keto 1 2 3 4 tetrahydro phenanthrene	50	28	1.25	30	c 0.2
20	"	9 10-dihydroxy 9 10 di n propyl 9 10 dihydro 1 2 5 6 dibenzanthracene	50	28	0.18	28	<0.01

21	Jensen sarcoma	12	18	1 16	14	c. 0.5
22	"	12	15	1 13	22	0.6-0.5
23	"	12	15	1.02	17	0.9
24	"	30	17	1.52	15	0.3-0.2
25	Walker carcinoma	50	34	1.34	28	0.1-0.05
25	"	50	34	0.98	25	>0.9
26	"	40	21	1.02	29	0.9-0.8
26	"	40	21	1.08	30	0.6-0.5
27	"	50	21	1.03	29	0.8-0.7
28	Rous sarcoma	90	18	1.36	16	0.3-0.2
29	Walker carcinoma	50	26	0.84	19	0.5-0.4
30	"	50	32	1.04	30	0.7-0.6
30	"	50	32	0.84	31	0.1
31	"	50	30	0.71	21	0.2
31	"	50	30	0.71	20	0.2
32	Benzpyrene-induced sarcoma (4th generation)	60	24	0.46	28	0.02-0.01
33	Benzpyrene-induced sarcoma (6th generation)	50	24	0.27	23	<0.01
34	Benzpyrene-induced sarcoma (5th generation)	50	26	0.20	17	<0.01
35	Dibenzanthracene-induced tumour LR-10 (87th generation)	30	17	0.31	29	<0.01

These results show a correlation between carcinogenicity and growth-inhibitory power, but this relationship is not quantitatively simple, since 1 2 5 6 dibenzanthracene proved to be more inhibitory than 3 4-benz pyrene, although their order of carcinogenicity is the reverse. If it be assumed that the two properties are connected etiologically this finding suggests various possibilities such as the following (a) that carcinogenicity is dependent on a certain optimal and not a maximal inhibiting power, or (b), that the relation between them is quantitatively simple but is disturbed by other factors such as the "toxicity" of individual compounds. Again while 6 methyl 1 2 benzanthracene is the most actively inhibitory (Exp 19) as well as the most carcinogenic among those methyl derivatives of 1 2 benzanthracene which were tested the 3 methyl and 7 methyl compounds also showed inhibitory properties (Exps 17, 18, 34) although they are classed with the non carcinogenic derivatives of 1 2 benzanthracene (Barry and others 1935). In any comparison between these biological properties it should also be borne in mind that while carcinogenicity has been determined mainly in mice these experiments on growth inhibition have been carried out almost exclusively with rats. Thus the statements made here about the carcinogenic or non carcinogenic, character of various compounds are derived chiefly from experiments upon the skin of the mouse. The accessibility of this particular test object is in a sense accidental, and its response, which is that of a single tissue in a single species may not give a complete picture of the property in question.

Figs 1-3 show that the effect of 1 2 5 6 dibenzanthracene was to produce an immediate and *prolonged* retardation in the growth rate even when the substance was administered in only two injections. In addition it can be seen that the retardation was relatively constant in any one experiment. It may also be noted that the control data are in agreement with the conclusions reached by Mayneord (1932) in the case of the Jensen sarcoma and Schrek (1935) for the Walker and Flexner Jobling carcinomata, that the unimpeded growth of these tumours is a linear function of the time over a considerable period.

The contrast between the growth inhibitory action of substances such as 1 2 5 6 dibenzanthracene and the inactivity of most of the non carcinogenic hydrocarbons is sufficiently striking but it is important to enquire if the action is characteristic. Prior to this work a number of experiments had been carried out to determine the effect on rat tumours of various toxic agencies such as anti rat precipitin and colchicine, and these experiments provided a useful standard by which to judge the significance of the inhibition produced by the carcinogenic hydrocarbons. Interference

with growth in such experiments was obviously due to a serious poisoning effect on the whole animal, and it was found that no lasting inhibition could be produced by amounts approaching the lethal dose. Fig. 13 illustrates the influence on the growth of the Jensen sarcoma of a suitable amount of anti-rat serum precipitin: temporary retardation was followed by rapid recovery. This action could not be intensified since larger doses proved highly toxic. In the case of colchicine some irregularity in growth rate was

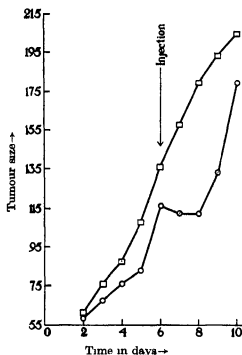


FIG. 13—○ Mean of three tumours treated with anti-rat precipitin, □ Control means of three tumours in rats given heat inactivated precipitin

produced by moderate doses but again it was impossible to increase or prolong the effect on account of the limits imposed by toxicity.

From these comparisons it appears that the prolonged retardation of growth rate produced by polycyclic hydrocarbons such as 1,2,5,6-dibenzanthracene, 3,4-benzpyrene and 5,6-cyclopenteno-1,2-benzanthracene must be regarded, so far as present knowledge goes, as a unique type of response to the administration of chemical substances.

Certain other changes were observed in tumours subjected to the action of the inhibitory hydrocarbons. Thus it was often noted that treated

tumours were firm in consistence as compared with their rapidly growing controls. Again microscopic examination showed some reduction in the proportion of dividing cells but no gross destructive changes. Dr P Ch Koller kindly examined some of the tumour material cytologically and confirmed the reduction in mitosis.

During certain experiments it was observed that the growth of the rats themselves was retarded. Although the degree of inhibition was much more striking in the case of the tumours it was concluded that the effect was in no sense specific for tumour tissue and that the latter simply shared in a retardation of growth affecting the body as a whole. This impression was fully confirmed in a separate series of experiments on the influence of carcinogenic hydrocarbons on body growth (Haddow, Scott and Scott 1937). The investigation also demonstrated the value of the young growing rat as a test object in work of this kind, since the material permits greater uniformity than can be attained with transplantable tumours even under the best conditions.

Apart from their action on body growth and tumour growth the carcinogenic hydrocarbons produced other effects which are no doubt correlated. In some experiments it was noted that the food intake of the treated rats appeared to be less than that of the controls, although their general health and activity remained good. The fertility of the treated animals was very definitely lowered, and in many cases the ovary showed suppression of ovulation and the testis a variable reduction of spermatogenesis (figs 14-15, Plates 23-24). In one case the administration of 50 mg 1,2,5,6-dibenzanthracene at the first appearance of the placental sign was followed by prolongation of the gestation period and death of the foetuses *in utero*.

Routine histological examination revealed no undoubtedly significant changes in the liver, kidney, spleen, suprarenal, thyroid or bone marrow. The influence of the active substances is thus specially evident in the case of growing tissues, and is unique in so far as doses which produce little sign of general toxicity or tissue damage may bring about a profound and continued alteration in the rate of growth. The phenomenon is clearly independent of toxic action in the non-specific sense, especially since recognized poisons such as colchicine evoke a different type of response. On the other hand it is equally true that the carcinogenic hydrocarbons tested have all shown some degree of toxicity. This manifested itself not by any immediate ill effect but rather by an increased susceptibility to infection which should perhaps be regarded as a secondary effect of the retardation of growth itself. Picard and Laduron (1934a, b) studied the toxicity of 3,4-benzpyrene in mice and found that doses of the order of

35 mg produced atrophic changes in the spleen thymus gland and lymph nodes More recently Polson (1936) has described experimental liver necrosis in the rabbit produced by the intraperitoneal injection of suspensions of 1 2 5 6 dibenzanthracene the results showed considerable variation and were not duplicated in the case of 3 4 benzpyrene

According to Zondek (1936 *a b*) prolonged administration of large doses of oestrin produces considerable inhibition of body growth the subcutaneous injection of 5000 m u dimenformon twice weekly in rats 4-6 weeks old retarded growth to the extent of nearly 45% after about 4 months The treated animals ate less food and Zondek referred to the possible interpretation that the animals eat less in consequence of the long treatment and are therefore retarded in their growth But in Zondek's opinion the intake is reduced because the animals have ceased growing and so require less food The growth inhibiting effect of oestrin was produced by percutaneous as well as subcutaneous administration and in birds as well as in rodents

Zondek also brought forward evidence that (*a*) prolonged administration of oestrin interferes with the anterior lobe of the pituitary (*b*) the process does not affect all the anterior pituitary functions equally and simultaneously since atrophy of the genitals is the first effect in the rat (*c*) elimination of the gonadotropic hormone is followed by failure of the growth hormone He regarded the stunting of growth produced by oestrin as a pituitary dwarfism since it could be abolished by giving an extract rich in growth hormone

In the present experiments the synthetic oestrogens 1 keto 1 2 3 4 tetrahydrophenanthrene and 9 10 dihydroxy 9 10 di *n* propyl 9 10 dihydro 1 2 5 6 dibenzanthracene behaved differently in their action on tumour growth the latter proving moderately inhibitory and the former inactive The former substance is non carcinogenic (Barry and others 1935) and the latter has given no tumours as yet although the experiments are incomplete (Burrows unpublished) It should also be noted that the oestrogenic activity of the keto compound is of an altogether lower order than that of the di *n* propyl diol (Cook and others 1934) Wolfe (1936) studied the action of this dibenzanthracene diol on the pituitary and found that it prevented the changes which occur after castration and also induced extreme degranulation of the basophiles and moderate degranulation of the eosinophiles In this comparison between the effects of carcinogenic and oestrogenic substances it may be noted that Cramer and Horning (1936) described arrest of cell division in the mouse testis after prolonged treatment with oestrin while Tuchmann (1936) also Tuchmann and Demay



1936) obtained similar regressive changes following the administration of either folliculin or 3 4 benzpyrene in guinea pigs

It is clear that at least a part of the general inhibition of growth produced by the carcinogenic hydrocarbons may be explicable on the basis suggested by Zondek for oestrin i.e. as an interference with pituitary function Nevertheless it also seems probable on account of the insolubility of the cancer producing compounds that in certain circumstances their action must be restricted to the cells in the immediate vicinity Tumour induction by means of the carcinogenic hydrocarbons is essentially a local process and this in itself indicates that such substances may act directly on exposed cells

#### *B—Interpretation and Significance*

These results show that the carcinogenic hydrocarbons possess unusual growth inhibiting properties and are not direct growth stimulating substances as has been assumed for instance by Needham (1936) We have thus a paradox in that the continued exposure of normal cells to a strongly inhibitory agent may result in the appearance of a rapidly growing malignant tumour The following hypothesis is put forward to explain this apparent contradiction

The available evidence points rather to the origin of cancer as a result of a change in cells at one time normal than to any selective process The cancer cell must therefore be regarded as a variant of its normal prototype from which it differs in metabolism and in the character of growth rate Moreover the study of its behaviour during prolonged transplantation shows that the *type* of variant it represents is discontinuous permanent and irreversible On these grounds it seemed that the origin of cancer could be resolved into one of the origin of variations in general and of discontinuous irreversible variations in growth rate in particular

In a study of variation in bacteria it was found that certain principles seemed to be applicable to the phenomena of variation in other types of cell Firstly the sources of variation were found to be mainly if not entirely environmental in origin Secondly for the purpose of inducing variation in respect of a given character there appeared to be two main requirements (a) a cell inherently capable of variation in respect of that character (b) a source of environmental interference with the character in question which is freely compatible with cell viability and so with the power to vary In particular it was found that variants characterized by permanently increased growth rate were produced not by any process of direct growth stimulation but rather as a sequel to a long continued period of growth inhibition It was concluded that when the growth of a potentially

variable organism is inhibited by a process which allows the majority of the affected cells to survive a relatively small number may undergo an irreversible change in their metabolism such that they can then multiply even in an environment which makes this difficult or impossible for their parent cell. The problem arose whether this might apply to the new cell race which constitutes a malignant tumour and whether the production of cancer might also be due to a sustained inhibition of the affected normal cells leading to the emergence of a new and advantageous variant with a permanently increased fission rate.

It is of interest that X and  $\gamma$  rays which in certain circumstances are associated with the induction of tumours (e.g. Ludin 1934, Ross 1936) have growth inhibiting powers to which they owe their main therapeutic application. This is specially significant since recent evidence (e.g. *Medical Uses of Radium*, M.R.C. 1934) tends to show that the effect of such agencies is inhibitory under all conditions of their action: low dosages and short non-lethal exposures producing a retardation of growth which is followed by recovery. The association of cancer with these radiations is therefore unlikely to be due to direct stimulation.

If the emergence of a chemically produced tumour is a response of normal cells to a long continued inhibition it will be of the greatest interest to determine the reaction of the new race of tumour cells to the hydrocarbon which induced their appearance. Exps. 32-5 give the only data at present available. It can be seen that the sarcoma induced by 3,4-benzpyrene was less sensitive to the action of the same compound than was the Walker carcinoma although it proved moderately sensitive to inhibition by 1,2,5,6-dibenzanthracene and 3-methyl-1,2-benzanthracene. The LR-10 sarcoma originally induced by 1,2,5,6-dibenzanthracene was fairly sensitive to inhibition by this compound when tested in the 87th generation of transplantation.

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perylene diphenylene oxide and 1 9 benzanthrone Dr P Ch Koller for a cytological examination of some of the tumours and Miss H H Smith for much help in connexion with the animals

#### IV—SUMMARY

1 A number of carcinogenic compounds including 1 2 5 6 dibenzanthracene 5 6 cyclopenteno 1 2 benzanthrone and 3 4 benzpyrene produced a considerable inhibition in the rate of growth of the Jensen and Walker tumours

2 This activity was also shown to a variable extent by chrysene and certain compounds of benzanthrone type the carcinogenicity of which is either feeble or nil

3 A series of related non carcinogenic compounds showed no inhibitory influence when tested under the same conditions

4 Of the synthetic oestrogens 1 keto 1 2 3 4 tetrahydrophenanthrene and 9 10 dihydroxy 9 10 di n propyl 9 10 dihydro 1 2 5 6 dibenzanthracene the latter proved to be inhibitory and the former quite inactive

5 These and others effects are discussed in relation to the mode of action of tumour producing substances in general

#### V—APPENDIX

*Exp 1 Jensen Sarcoma Individual Tumour Measurements (length × breadth × thickness in cm) Injections from 10th Day after Implantation*

Days after implantation	Dibenzanthracene colloid daily				Control			
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8
10	4.7	4.2	3.9	2.8	7.3	3.5	3.7	1.8
11	4.4	5.6	5.1	3.8	7.6	5.6	5.2	2.2
12	6.9	8.2	7.3	4.6	14.2	8.2	8.0	3.2
13	8.2	11.0	9.3	5.6	21.0	13.3	12.7	4.8
14	11.9	14.7	10.6	7.3	27.5	17.6	16.7	6.5
15	12.6	20.0	12.7	7.3	31.4	22.8	21.2	8.6
16	17.2	23.0	13.2	9.5	27.0	30.0	25.0	10.4
17	21.2	20.5	13.4	10.9	38.7	36.9	28.7	12.8
18	19.9	22.3	13.0	9.4	46.0	35.2	31.8	15.1
19	20.2	26.3	15.6	10.6	46.5	45.0	42.2	16.6
20	23.2	25.0	17.2	11.2	47.8	42.5	40.0	20.8
21	23.2	31.9	16.3	12.5	57.1	52.0	Died	26.6
22	26.4	29.5	17.5	13.5	90.3	55.5	—	33.2
23	21.2	33.0	19.7	12.9	75.0	62.2	—	38.6
24	20.5	27.9	19.5	15.0	80.5	60.2	—	43.3

Exp 3 Individual Tumour Dimensions (length  $\times$  breadth in cm)

Treatment on 10th and 11th days	Rat no	Days after implantation										
		5	6	7	8	9	10	11	12	13	14	15
Nil	1	2.5	4.0	4.6	5.4	5.0	5.4	6.8	7.2	7.6	8.0	8.7
	2	1.1	2.1	2.7	5.4	8.1	7.3	8.1	7.8	7.8	8.6	9.6
	3	4.0	4.9	7.9	9.9	11.0	10.8	12.6	13.8	13.0	15.6	16.0
	4	3.0	4.2	5.4	5.6	8.4	7.6	9.4	10.1	11.0	11.2	14.6
5 cc sesame oil	5	2.0	2.6	3.9	5.0	5.9	6.6	7.3	8.2	8.6	10.3	10.6
	6	3.8	4.9	6.6	6.6	8.5	9.6	10.4	9.0	12.7	12.3	12.0
	7	2.6	2.6	3.4	4.4	4.9	5.9	5.9	6.3	6.8	8.7	10.5
	8	3.8	4.0	4.2	4.8	6.5	7.3	8.0	9.6	7.7	8.4	7.9
	9	4.3	5.3	6.9	8.7	10.8	13.3	12.7	12.5	15.2	16.5	19.2
	10	4.3	5.6	8.1	8.1	13.0	12.4	13.9	10.4	13.0	16.6	18.0
25 mg 1 2 5 6 dibenzan thracene	11	2.0	2.9	3.5	2.8	3.9	4.2	4.2	3.7	3.8	3.8	3.9
	12	3.1	6.4	8.0	9.9	11.1	12.7	12.7	12.4	11.3	10.4	9.2
	13	2.3	3.3	4.0	4.4	5.9	5.4	5.1	5.4	4.9	5.4	5.6
	14	2.0	2.8	3.3	5.5	6.3	5.8	7.0	6.9	5.4	5.5	5.6
	15	3.9	5.7	7.0	8.4	10.5	10.6	10.7	10.5	11.0	9.6	8.1
	16	3.2	4.2	6.9	6.0	7.0	7.6	7.4	7.3	6.6	7.1	6.4

		Days after implantation									
		16	17	18	21	23	25	29	31	35	44
Nil	1	9.7	10.4	11.2	16.2	21.6	21.6	35.0	39.5	43.0	Died
	2	11.1	11.4	12.4	17.0	21.6	22.8	33.2	32.8	33.6	36.0
	3	16.6	19.0	21.2	24.6	23.2	26.6	30.6	27.2	34.2	Died
	4	12.2	13.9	18.1	24.1	29.2	32.0	30.8	35.6		Died
5 cc sesame oil	5	11.4	13.2	15.7	19.2	26.3	Died				
	6	14.7	17.0	18.3	19.0	26.9	28.5	26.9	32.8	31.1	26.2
	7	11.3	11.2	13.9	17.3	19.0	19.7	28.1	30.0	29.6	Died
	8	9.0	9.2	10.2	14.3	14.9	16.6	19.6	17.7	17.0	Died
	9	17.8	19.9	21.4	24.3	26.6	30.0	Died			
	10	16.3	18.6	19.5	26.2	32.0	30.1	Died			
25 mg 1 2 5 6 dibenzan thracene	11	4.0	4.0	5.0	5.1	5.2	3.8	3.5	3.6	2.9	3.2
	12	9.2	10.2	9.7	8.6	9.8	9.5	9.4	9.0	9.5	8.4
	13	5.6	5.6	6.0	7.4	7.7	6.7	6.9	6.6	8.8	8.2
	14	5.6	5.7	4.9	6.5	5.7	5.1	6.3	7.4	8.1	10.6
	15	9.4	9.7	9.7	9.0	8.6	8.8	8.2	9.5	10.6	9.0
	16	5.6	5.9	6.6	6.4	8.0	7.2	7.8	8.4	7.8	10.1

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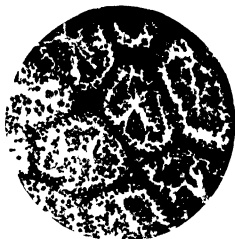
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## DESCRIPTION OF PLATES 23, 24

FIG. 14—Testes from rats given A, 50 mg. 1,2,5,6-dibenzanthracene; B, 50 mg. pyrene 3 weeks previously.  $\times 105$ . Haematoxylin and eosin.

FIG. 15—Ovaries from rats given A, 50 mg. 6-methyl-1,2-benzanthracene; B, sesame oil alone 6 weeks previously.  $\times 24$ . Iron haematoxylin



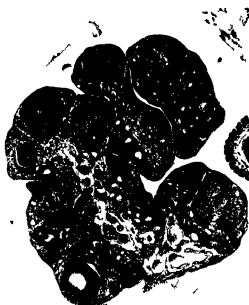
A



B

Fig. 14

$\times 10$



A



B

FIG. 15

× 24

# The Influence of Certain Carcinogenic and Other Hydrocarbons on Body Growth in the Rat

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## I—INTRODUCTORY

In the course of work on the mode of action of tumour-producing agents it was found that certain of the carcinogenic hydrocarbons studied at the Research Institute of the Royal Cancer Hospital (Cook and others 1932, Cook 1932, Barry and others 1935) possessed growth-inhibiting properties (Haddow 1935, Haddow and Robinson 1937). This was demonstrated by recording the effect of these substances, administered by intraperitoneal injection, on the behaviour of transplantable tumours growing subcutaneously in the rat. During these experiments it was observed that treated animals appeared to grow less rapidly as compared with their controls. Because of this it was felt that the reduction in tumour growth rate was probably not a tumour-specific effect but rather the result of an inhibition of growth affecting the body as a whole. Hence further experiments were carried out to determine the action of these and other substances on the body growth of rats.

## II—EXPERIMENTAL

1—*Animals*—The animals used were from an inbred colony of hooded rats of the Lister strain, maintained under standard conditions of housing and feeding. The diet was one of bread and milk (alternating with biscuit and milk) with additions of cod-liver oil and marmite, fresh greens, whole oats and water were also supplied daily. The room temperature was kept at 65–70° F.

2—*General Design of Experiments*—Rats to be used for a given experiment were obtained from several litters (usually four but occasionally five)

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born within a space of 48–72 hr, when about 55 days of age they were ear marked and weighed individually each day for at least a week. This procedure indicated the rate of increase and its variation, and also enabled the animals to adjust themselves to handling. At the end of this preliminary control period they were arranged in groups, special care being taken to ensure that each of these contained, as far as possible, an equal share from every litter and the same proportion of males and females. The following data show that the groups agreed closely not only in composition and sex ratio but also in their initial mean weight and rate of growth. The majority of the experiments contained three such balanced groups so that two substances might be tested simultaneously with a common control.

The rats in each set were then injected with the appropriate compound usually in a single dose but occasionally in two, while the controls received an equal volume of the solvent alone. The individual litters were reconstituted immediately after injection and kept in separate cages to the end of the experiment. Individual daily weighings were then continued for as long a period as was thought to be necessary.

3—*Technique of Administration*—All the hydrocarbons and related substances were dissolved in sesame oil, in the hot air oven at about 100° C. in concentrations of 0.5 or 1.0%. The resultant sterile solutions were injected intraperitoneally. Other substances (*vide infra*) were dissolved in suitable concentration in sterile water or saline and administered by the same route.

4—*Recording of Data*—A complete record was kept of the individual data from day to day and graphs were also constructed to indicate the rate of increase of each group as a whole. For the latter purpose it was found necessary to apply corrections in certain cases in respect of (a) the death of animals, and (b) the occurrence of pregnancy. These adjustments were in no case serious and they are indicated where required in the tables which follow. It should be pointed out that the animals used were of such an age as to enable conclusive results to be obtained before the first pregnancies occurred. In some experiments, however, the observations were continued well into the period of sexual maturity, and in these cases the data for males alone were employed.

The mean figures at certain selected periods of different experiments are presented in tabular form, while the individual data on which these are based are given in the Appendix. The whole course of each experiment is also shown in graphs (figs. 1–11).

*A—Hydrocarbons and Related Substances*

*Exp 1—1 2 5 6-Dibenzanthracene, pyrene*—The first experiment was planned to determine the action on normal growth of a carcinogenic hydrocarbon (1 2 5 6 dibenzanthracene) and a non carcinogenic compound (pyrene) These were selected as representative of substances which had already been studied in the work referred to above, when 1 2 5 6 dibenzanthracene proved to be inhibitory (as tested on the growth of the Jensen and Walker tumours) and pyrene to be quite inactive in this respect.

Forty rats (46–48 days old) were used from four litters of 12, 11, 10 and 8 After a preliminary control period they were divided into three groups as follows Groups I and II were composed of four animals from each of two litters and three from each of the remaining two, while group III contained two animals from one litter, four from another, and three from each of the remaining two The groups were also adjusted so as to contain comparable numbers of males and females

On the 8th day the controls each received 2 c c sterile sesame oil, those in the second group 10 mg 1 2 5 6 dibenzanthracene, and those in the remaining group 10 mg pyrene, in 2 c c oil

Table I summarizes the position at the 43rd day, fig 1 indicates the respective growth rates in the three groups to the 48th day, and fig 2 shows the relative distribution of individual weights at the 1st, 8th and 100th days in the male animals surviving thus far

TABLE I

Group	Treatment on 8th day	Mean body weight (g )			Percentage increase in weight	
		1st day	8th day	43rd day	1st–8th day	8th–43rd day
I	2 c c sesame oil i p	56.4	69.0	133.5	22.3	93.4
9 ♂ 5 ♀						
II	10 mg 1 2 5 6 dibenz anthracene in oil i p	53.8	66.5	97.0	23.6	45.8
9 ♂ 5 ♀						
III	10 mg pyrene in oil	55.0	67.5	132.7	22.7	96.5
8 ♂ 4 ♀	i p					

Pyrene under these conditions was totally without influence on the body growth, while the same dose of 1 2 5 6 dibenzanthracene produced an immediate and prolonged retardation in the growth rate amounting to 50% of the normal and continuing, with no indication of recovery, at least for 15 weeks

In view of this result it was decided to study the influence of varying dosages of 1 2 5 6 dibenzanthracene

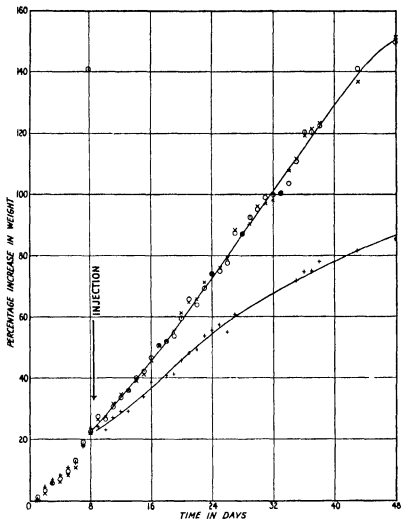


FIG. 1—x Control oil, 2 c c , o control pyrene, 10 mg in oil,  
+ 1 2 5 6 dibenzanthracene, 10 mg in oil

*Exp 2—1 2 5 6 Dibenzanthracene*—After preliminary weighing for 7 days, four litters of 12, 9, 7 and 7 rats were used to provide three balanced groups as before. Animals in one group then received two injections (separated by an interval of 2 days) of 15 mg 1 2 5 6-dibenzanthracene in 3 c c sesame

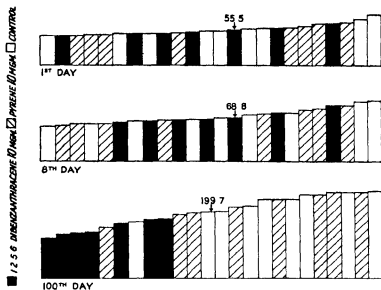


FIG 2—Relative distribution of individual weights for male animals in Exp 1 (treatment on 8th day) Arrows indicate position of mean weight (g)

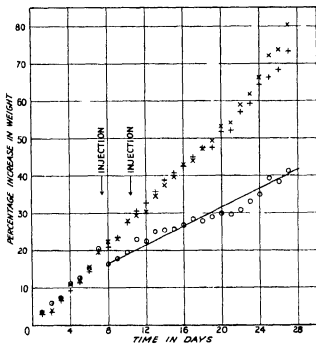


FIG 3—x Control untreated + control (2 x 3 cc oil),  
o 1,2,5,6 dibenzanthracene, 2 x 15 mg

oil those in the second received similar injections of oil alone while the third group remained an entirely untreated control

Fig 3 shows the course of events to the 26th day and Table II the data for the male animals to the 38th day

Group	Treatment on 8th and 11th days	TABLE II Mean body weight (g)			Percentage increase in weight	
		1st day	8th day	38th day	1st-8th day	8th-38th day
I 5 ♂	Control untreated	60.4	72.8	121.8	20.5	68.0
II 6 ♂	Control oil 2 × 3 c.c. i.p.	68.8	83.1	137.1	20.8	64.9
III 5 ♂	1.2.5.6 dibenzanthracene 2 × 15 mg i.p.	65.6	78.8	108.6	20.1	37.8

Although the inhibitory effect was not increased in simple proportion to the increase in dose of hydrocarbon the result clearly confirms the first experiment and shows that the injection of sesame oil alone was quite without influence on the normal rate of growth

*Exp 3 (part I)—1.2.5.6 Dibenzanthracene*—Two of three groups made up from three litters of 9, 8, 6 and 5 animals were used to determine the inhibitory effect of 90 mg 1.2.5.6 dibenzanthracene. After preliminary weighings the rats received two intraperitoneal injections (at an interval of 3 days) of 4.5 c.c. of a 1% solution of 1.2.5.6 dibenzanthracene in sesame oil. Fig 4 shows the extent of the inhibition and its duration to the 40th day. In this case the maximal effect was delayed as compared with the previous experiments and it was felt that this was due to irregular absorption: a 1% preparation of the hydrocarbon was employed in order that sufficient might be contained in a volume suitable for injection and as some crystallization occurred at least a part of the dose must have been relatively inert for some time after administration. Table III gives the

Group	Treatment on 8th and 11th days	TABLE III Mean body weight (g)			Percentage increase in weight	
		1st day	8th day	26th day	1st-8th day	8th-26th day
I 2 ♂ 7 ♀	Control oil 2 × 4.5 c.c. i.p.	64.0	76.0	107.1	18.7	40.9
II 3 ♂ 6 ♀	1.2.5.6 dibenzanthracene 90 mg i.p.	65.3	77.0	83.8	17.9	8.8

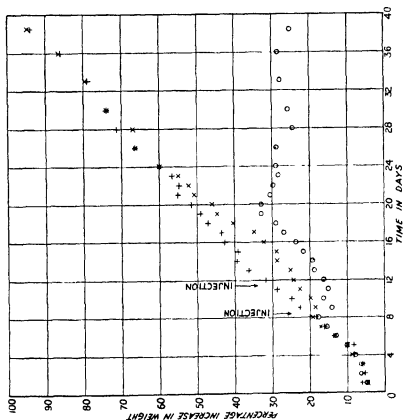


FIG. 4—x X-rayed—450 r, 0-01 Al screen, + control (out),  
 o 1,2,5,6 dibenzanthracene, 90 mg

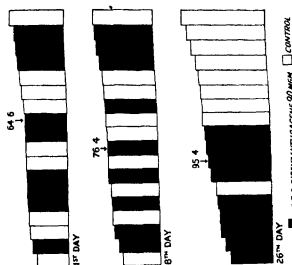


FIG. 5—Relative distribution of individual weights in Exp 3 (treatment on 8th and 11th days). Arrows indicate position of mean weight (g)

data for the 1st, 8th and 26th days of this experiment, while fig 5 indicates the highly significant change in the random array order of individual weights which took place as a result of the treatment

*Exp 4—1 2 5 6 Dibenzanthracene*—When the inhibiting effect of 1 2 5 6-dibenzanthracene on body growth had been demonstrated for doses of 10, 30 and 90 mg, an experiment was carried out to test the influence of 3 mg under the same conditions. Twenty eight rats were used and fig 6 shows the course of the experiment during a month. It will be seen that there is an undoubtedly significant result. Table IV gives the details for

TABLE IV

Group	Treatment on 8th day	Mean body weight (g)			Percentage increase in weight	
		1st day	8th day	34th day	1st 8th day	8th-34th day
I	Control oil	58.3	74.3	116.5	27.5	57.0
7 ♂						
II	1 2 5 6 dibenzanthra- cene, 3 mg in oil	59.8	77.3	113.0	29.1	46.2
7 ♂						

the male animals at the 1st, 8th and 34th days, and it seems that this effect, although much less than in the previous experiments, is probably of the same type, i.e. an alteration in the rate of growth.

*Exp 5—1 2 5 6 Dibenzacridine, chrysene*—Thirty animals from five litters of 8, 7, 7, 4 and 4 were used, in adjusted groups of ten, to compare a hydrocarbon of doubtful carcinogenicity (synthetic chrysene) with a moderately carcinogenic substance (1 2 5 6 dibenzacridine). These were given in a single dose of 30 mg in 1% solution in oil. Fig 7 shows the results up to the 38th day. It is clear (a) that chrysene led to a significant departure from the normal growth curve, and (b) that 1 2 5 6 dibenzacridine produced a considerable alteration in growth rate. In the latter case this change was preceded by a fall in weight in the 36 hr following injection. On this account the percentage increase in weight after treatment (see Table V) was calculated from the 13th day and not from the date of injection as in previous experiments. The figures also show that chrysene in this dosage probably produced no lasting alteration in the growth rate.

*Exp 6—Dodecahydro 1 2 benzanthracene, fluoranthene*—This experiment was designed to test the action on body growth of two non-carcinogenic compounds, which had been shown by previous work to be devoid of

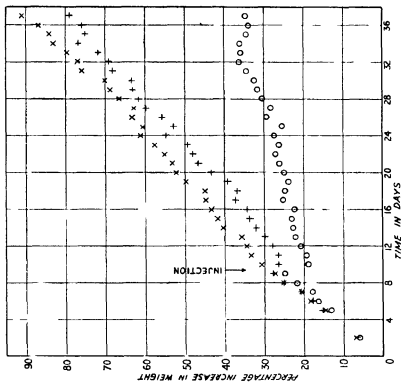


FIG 7—x Control (3 c oil), + chrysene 30 mg,  
o 1 2 5 6 dibenzacridine 30 mg

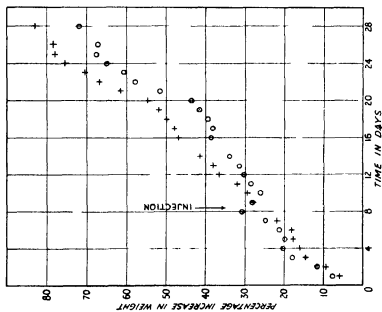


FIG 6—+ Control (oil), o 1 2 5 6 dibenzanthracene, 3 mg



TABLE V

Group	Treatment on 8th day	Mean body weight (g)				Percentage increase in weight	
		1st day	8th day	13th day	30th day	1st-8th day	13th-30th day
I	Control oil,	76.2	90.7	100.1	126.3	19.03	26.17
4 ♂ 6 ♀	3 c c						
II	30 mg ehry sene in 3 c c	76.3	92.0	95.2	121.9	20.05	28.04
4 ♂ 6 ♀							
III	30 mg 1 2 5 6 dibenzacridine in 3 c c	76.3	88.7	86.3	92.7 (corrected to allow for 2 deaths)	16.25	7.41
4 ♂ 6 ♀							

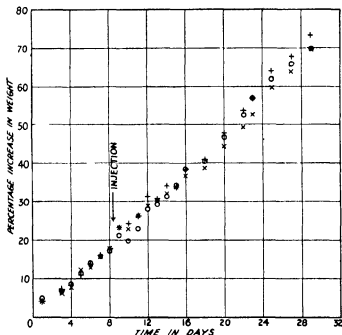


FIG 8—x Control (3 c c oil) + dodecahydro 1 2 benzantracene, 30 mg ,  
 o fluoranthene, 30 mg

inhibiting influence on the growth of the Walker carcinoma. The substances, dodecahydro 1 2 benzantracene and fluoranthene, were given in a dose of 30 mg to two groups (fig 8, Table VI). The rats were derived from four litters of 9, 8, 6 and 4 animals. Under the conditions stated, the compounds employed had no effect of any kind on the normal course of body growth.

TABLE VI

Group	Treatment on 8th day	Mean body weight (g)			Percentage increase in weight		
		1st day	8th day	24th day	1st-8th day	8th-24th day	
I	3 c c oil	68.1	80.1	108.5	17.6	35.5	
3 ♂ 6 ♀							
II	30 mg dodecahydro	70.7	84.4	115.7	19.2	37.1	
4 ♂ 5 ♀	1.2 benzanthracene 1 %						
III	30 mg fluoranthene	70.1	82.1	113.4	17.1	38.1	
4 ♂ 5 ♀							

*Exp 7*—1.2.5.6 Dibenanthracene 1.2.5.6 dibenzacridine 3.4 benzpyrene  
—Exp 7 served to compare two substances already dealt with (1.2.5.6 dibenzanthracene and 1.2.5.6 dibenzacridine) with each other and with another carcinogenic hydrocarbon (3.4 benzpyrene) previously shown to have an inhibitory influence on the growth of transplanted tumours in the rat

Animals from litters of 11, 10, 10 and 5 were divided to give four strictly comparable groups as before. After preliminary weighings these received injections of control oil, 10 mg 1.2.5.6 dibenzanthracene, 10 mg 1.2.5.6 dibenzacridine or 10 mg 3.4 benzpyrene (fig. 9 and Table VII). It will be

TABLE VII

Group	Treatment on 8th day	Mean body weight (g)				Percentage increase in weight		
		1st day	8th day	10th day	30th day	1st-8th day	8th-10th-30th day	
I	2 c c sesame oil i p	66.7	80.1	81.8	120.7	19.9	47.5	
4 ♂ 5 ♀					(1 animal omitted on account of pregnancy)			
II	10 mg 1.2.5.6 dibenzacridine in oil	64.6	78.1	75.2	97.4	20.9	29.5	
4 ♂ 5 ♀					(1 sick animal omitted)			
III	10 mg 1.2.5.6 dibenzanthracene in oil	69.4	84.4	84.4	102.0	21.5	20.8	
4 ♂ 5 ♀					(1 sick animal omitted, 1 death)			
IV	10 mg 3.4 benzpyrene in oil	66.9	80.7	78.1	105.3	20.6	34.8	
4 ♂ 6 ♀								

seen (a) that 1 2 5 6 dibenzanthracene produced an effect almost identical with that obtained in Exp 1, (b) that the response to 1 2 5 6 dibenzacridine was of the same type as in Exp 5, i.e. an initial fall in weight succeeded by resumption of growth at an altered rate, and (c) that while

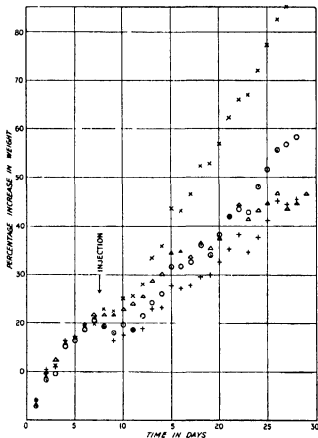


FIG 9—x Control (20 c.c. oil), + 1 2 5 6 dibenzacridine 10 mg  
 Δ 1 2 5 6 dibenzanthracene, 10 mg, ⊙ 3 4 benzpyrene, 10 mg

3 4 benzpyrene produced the same initial effect as 1 2 5 6 dibenzacridine, the subsequent inhibition of growth was less than in the case of 1 2 5 6 dibenzacridine or 1 2 5 6 dibenzanthracene

#### B—Miscellaneous Agencies

The results already described seemed to confirm the original impression that the carcinogenic compounds employed possessed an inhibitory

influence on body growth in the rat which depended upon a prolonged reduction in growth rate. At this stage it seemed desirable to carry out further experiments to test and compare the effect of certain miscellaneous agencies likely to interfere with normal growth.

*Exp 3 (part II)—X radiation*—In *Exp 3 (vide supra)* a group of ten animals was used to test the action of a suitable exposure to X radiation. On the 8th day these received a dose of 450 r through a screen of 0.01 mm aluminium; the radiation used was very soft, the equivalent wave length being about 0.4 Å. This produced an immediate interference with growth which reached its maximum about a week later; recovery then commenced and was complete within three weeks from the exposure (fig. 4, Table VIII).

TABLE VIII

Group	Treatment on 8th day	Mean body weight (g)			Percentage increase in weight			
		1st day	8th day	14th day	1st day	8th day	8th day	14th day
I	Control	64.0	76.0	89.4	18.7			17.6
2 ♂ 7 ♀								
II	X irradiation (450 r)	63.7	76.1	82.1	19.5			7.9
3 ♂ 7 ♀								

*Exp 8—Lead Nitrate*—Twenty-six animals from four litters were arranged in two groups. In this case the preliminary weighings were continued to the 15th day. Each rat then received an intraperitoneal injection of 4 mg lead nitrate in water while the others received a corresponding volume of the solvent alone (fig. 10). The administration of lead produced an immediate retardation which was soon followed by resumed growth at a rate identical with that of the controls (Table IX).

TABLE IX

Group	Treatment on 15th day	Mean body weight (g)				Percentage increase in weight	
		1st day	15th day	18th day	34th day	1st-15th day	18th-34th day
I	4 mg lead nitrate	56.7	84.5	85.3	112.5	48.9	31.8
7 ♂ 6 ♀							
II	Control	56.7	86.7	92.3	121.8	52.7	32.0
6 ♂ 7 ♀							

*Exp 9 (part I)—Colchicine*—Four litters of 10, 9, 8 and 6 animals served to give three comparable groups, and two of these were used to test the

action of colchicine After control weighing for 12 days, the experimental animals received 0.03 mg colchicine in saline intraperitoneally and those of the control series an equal volume of saline alone. The result (fig 11)

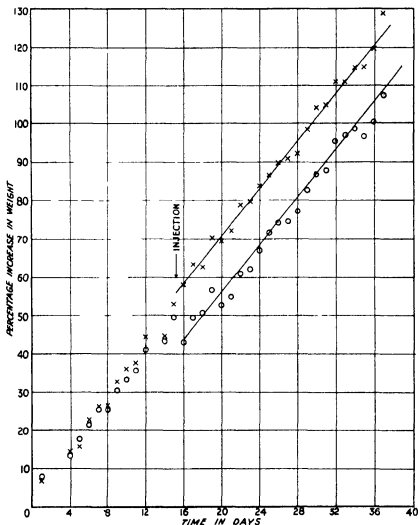


FIG 10—x Control, o lead nitrate, 4.0 mg

was a slight but probably significant drop in weight followed by immediate recovery

A second injection was then given of twice the initial dose. This in turn produced an undoubtedly significant retardation followed by slow recovery

over a period of 5-6 days. When this seemed complete a third dose of 0.12 mg. was administered and controlled in the usual way. In this amount the alkaloid proved manifestly toxic and led to a considerable fall in

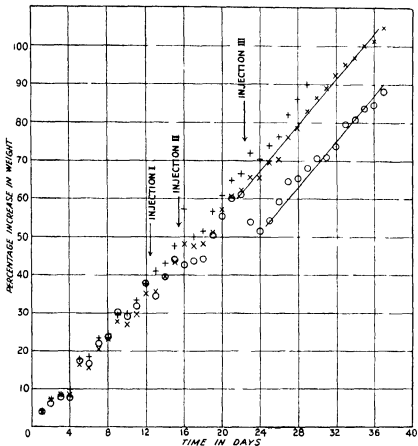


FIG 11—x Control (saline), + phenobarbitone I, 5 mg, II, 7.5 mg, III, 10 mg, o colchicine I, 0.03 mg, II, 0.06 mg, III, 0.12 mg

weight (fig. 11) Within 3 days, however, growth was resumed and thereafter proceeded, to the end of the experiment on the 37th day, at approximately the same rate as that of the controls (Table X).

*Exp. 9 (part II)—Phenobarbitone*—A third group was included in Exp. 9 in order to determine the influence on growth of phenobarbitone, using the same controls. Three successive injections were given of 5.0, 7.5 and 10.0 mg, dissolved in saline, on the same dates as the injections of colchicine in

TABLE X

Group	Treatment on 22nd day	Mean body weight (g)				Percentage increase in weight		
		1st day	22nd day	25th day	36th day	1st-22nd day	25th day	36th day
I 6 ♂ 5 ♀	0.5 cc saline i.p.	65.7	106.4	111.1	130.6 (1 animal omitted on account of pregnancy)	61.9		17.5
II 5 ♂ 6 ♀	0.12 mg col chicine i.p.	64.3	103.3	101.6 (3 animals dead)	121.7	60.7		19.8

part I of the experiment (above). Although these doses were sufficient to produce mocoordination and anaesthesia it is clear from fig 11 and Table XI that they had at least no inhibitory influence on growth.

TABLE XI

Group	Treatment	Mean body weight (g)		Percentage increase in weight 1st-36th day
		1st day	36th day	
I 6 ♂ 5 ♀	Saline control	65.7	130.6 (1 animal omitted on account of pregnancy)	98.7
II 6 ♂ 5	Phenobarbitone (see text)	64.8	136.7 (2 animals omitted on account of pregnancy)	110.9

### III—DISCUSSION

#### A—The Carcinogenic Compounds

The above results (Exps 1 2 3 4 5 7) show that the administration to young rats of certain carcinogenic substances (1 2 5 6 dibenzanthracene 1 2 5 6 dibenzacridine 3 4 benzpyrene) produced a profound alteration in growth rate. From figs 1 3 4 6 7 and 9 it is also apparent that this response occurred at once and that the degree of inhibition was approximately constant to the end of the period of observation. The change following a single injection was extremely prolonged if not permanent and no recovery was observed in any of the experiments described. It may be convenient at this point to discuss the phenomenon in greater detail as regards its nature and persistence.

An important question is the relationship of the inhibitory response to *toxic action*. Experience gained in the present work has shown that, while the carcinogenic substances employed possess definite toxicity, this is of a special type. In the first place, healthy animals may tolerate relatively large doses without immediate ill effect—although an initial drop in weight may occur in certain cases—and the organs of such animals need show no gross or microscopic evidence of damage. On the other hand spermatogenesis and ovulation may be diminished (Haddow and Robinson 1937) and clear evidence was obtained in the present work of a considerable lowering of both male and female fertility after administration of 1 2 5 6 dibenzanthracene. Again, the impression has been formed that these carcinogenic substances may prove indirectly harmful by lowering the bodily resistance to any existing source of infection. It is of interest that a pulmonary infection was the apparent cause of death in every animal—control or treated—which died in the course of these experiments. An indication of the number of deaths can be gained from Table XIII in the Appendix: mortality was considerably higher in animals receiving carcinogenic substances (particularly 1 2 5 6 dibenzacridine), but in many cases death occurred only after a fair period following injection.

Since then direct toxic action appears slight in relation to dose it seems that the growth inhibitory properties of the carcinogenic hydrocarbons, while not necessarily specific, are clearly not dependent in a non specific sense on mere toxicity alone. This conclusion is further supported by the fact that acknowledged poisons such as lead and colchicine produce an entirely different type of effect.

Considerable interest also attaches to the means by which the alteration in growth rate is prolonged, say for several months, following a single injection. The simplest possibilities are (a) that such substances acting on dividing cells produce a change of growth rate which persists after the agent itself has been removed or (b) that the effect is due to the continuous action of the substance itself or of a derivative. The data available can be readily summarized, although at present they are insufficient to yield an answer to this important question.

Chalmers (1934) determined the quantity of 1 2 5 6 dibenzanthracene remaining in the breast muscle of fowls at short intervals after local injection of the hydrocarbon (1–5 mg.) dissolved in chicken fat or egg yolk fat. The substance disappeared rapidly from the site of injection, decreasing to less than one tenth of the original quantity within a few days. Chalmers and Peacock (1936) found that 3 4 benzpyrene and 1 2 5 6 dibenzanthracene were eliminated from chick embryo within a few days of the



intramuscular injection in fatty solution of fractions of a milligram of these substances. Minute quantities of the former compound were also removed within a few hours of the intravenous injection of colloidal preparations in both chick embryos and mice. Berenblum and Kendal (1936) estimated the concentration of 1 2 5 6 dibenzanthracene at different times after the intraperitoneal injection of 2 mg. in mice. The greater part disappeared within 3 weeks and the loss was more complete when the substance was injected dissolved in lard than when it was given as a colloidal solution in water.

The mode of action of these substances is certainly complex. Peacock (1936) stated that during the elimination of 3 4 benzpyrene from the blood of fowls, rabbits and guinea pigs, the bile showed fluorescence due not to benzpyrene itself but to a derivative or derivatives. Again the urine of rabbits which had received colloidal benzpyrene, dibenzanthracene or anthracene intravenously was found to contain an ether-soluble fluorescent substance not normally present; this indicated that some water-soluble product might be reabsorbed from the gut and excreted by the kidney.

In the course of the present work Dr I. Hieger kindly examined several animals from Exps 1, 2 and 4. It was found that rats which had received 30 mg. of 1 2 5 6 dibenzanthracene in oil retained some of the hydrocarbon after 40 days. On the other hand, animals which had been injected with doses of 10 and 3 mg. showed no evidence of the presence of 1 2 5 6 dibenzanthracene after 105 and 37 days respectively, i.e. at a time when the inhibitory effect in these experiments was still maintained. The sensitivity of the method of assaying of the hydrocarbon is at present under investigation. Such results are suggestive although further work is obviously required.

#### *B. Comparison with other Substances and Agencies*

1—*Related Non-Carcinogenic Compounds*—In contrast to the above mentioned carcinogenic substances it is clear that the related non-carcinogenic compounds studied were quite inactive. When taken together these results indicate a certain parallelism in substances of this class between carcinogenicity and the power to bring about a long continued alteration in the rate of growth. In this respect the case of synthetic chrysene is of special interest as that of a substance in which carcinogenicity is doubtful or low and inhibiting potency correspondingly weak. It can be seen from Exp. 5 that 30 mg. chrysene gave a response rather less than that produced by only 3 mg. 1 2 5 6 dibenzanthracene.

On the other hand although the carcinogenic activity of 3 4 benzpyrene is much greater than that of 1 2 5 6 dibenzanthracene which in turn is more potent than 1 2 5 6 dibenzacridine (Barry and others 1935) Exp 7 shows that the inhibitory power of these substances is in the reverse order This would suggest if carcinogenicity and inhibiting power are intimately connected that the former is dependent on an *optimal* degree of inhibition of growth rather than a maximal other possible factors being equal It should also be borne in mind however that the carcinogenicity of such substances has mostly been studied in mice the inhibitory influence exclusively in rats

The growing rat adequately controlled appears to be an excellent test object for the estimation of inhibitory power The degree of accuracy attainable is shown by comparing two identical experiments carried out at an interval of about 6 months whereas in Exp 1 (after administration of 10 mg 1 2 5 6 dibenzanthracene) the ratio growth rate of treated animals growth rate of controls was as 458 934 i e 0 49 the same ratio for the control and dibenzanthracene animals in Exp 7 was 208 475 i e 0 44 In this paper it is not proposed to discuss further the etiological connexion which may possibly exist between carcinogenicity on the one hand and inhibitory power on the other (see Haddow and Robinson 1937)

2—*Toxic Agencies*—Exp 3 (part II) was included in order to study the retardation of growth produced by a single measured exposure to X radiation The growth rate was temporarily lowered but soon recovered after passing through two periods during which it was successively normal and then greater than normal No experiments have yet been carried out in this work to depress the growth rate continuously by suitably spaced exposures

Lead nitrate was investigated as an example of a heavy metal having toxic properties which often manifest themselves by producing interference with growth The substance was of additional interest as one which is excreted relatively slowly The dose given (approximately 50 mg/kg) was selected as likely to produce a detectable response without serious poisoning and corresponded to about one fifth of the amount found by Buck and Kumro (1930) to produce death after intraperitoneal administration It has already been noted that the treatment merely resulted in a temporary setback and was entirely devoid of any lasting influence on the rate of growth which became normal within a few days

It was thought desirable to investigate the influence of the alkaloid colchicine as a substance possessing not only general toxicity but also

special activity as a mitotic poison The work of Lits (1934) Dustin (1934) Brues and Cohen (1936) and Ludford (1936) has shown beyond all doubt that colchicine produces an arrest of cell division in metaphase which persists for several hours after administration This phenomenon—the basis of the so called caryoclastic crisis—occurs both *in vivo* and *in vitro* and is most obvious in tissues in which the mitosis rate is normally high such as the glands of Lieberkuhn (Dustin 1934) the regenerating rat liver (Brues 1936) and various transplantable animal tumours

The smallest amount employed in the present study (Exp 9 part I) corresponded closely to the minimum effective dose (0.02 mg/100 g) described by Brues and Cohen (1936) as the smallest dose which gave obvious abnormalities of mitosis in regenerating liver As has already been noted above the interference with growth produced by this amount was barely significant When the dose was doubled a few days later however retardation was immediate and pronounced This in turn was succeeded by recovery both to the original rate of growth and the expected level of weight The quantity of colchicine given in the third injection was identical with the optimal dose (0.1 mg/100 g) of Brues and Cohen—defined by these workers as the average amount giving a maximal number of abnormal mitoses—and equal to 20 per cent of the average lethal dose The result was a cessation of growth lasting only for a few days and followed by resumption at the normal rate It is important to note that this amount of colchicine caused death in three of eleven animals within 72 hr of injection

The second part of Exp 9 showed that the doses of phenobarbitone employed had certainly no inhibitory effect on growth Such negative evidence is of obvious interest in relation to the significance to be attributed to the types of growth disturbance already described

3—*Retardation of Growth Produced by Deprivation of Vitamins and Protein*—Osborne and Mendel (1914) in experiments in which prevention of growth was attained by a variety of dietary methods showed that the capacity to grow remained unaffected Later (1915) they quoted several remarkable instances of this phenomenon some of which are collected in Table XII

In this paper Osborne and Mendel wrote It should be noted in connexion with the foregoing individuals that their curve of growth after the period of suppression was as a rule comparable with that of a growing rat of the same size and sex The usual rate of body increment was not diminished but if anything was sometimes somewhat accelerated during

TABLE XII (AFTER OSBORNE AND MENDEL 1915)—CAPACITY OF ALBINO RATS TO GROW AT A VERY LATE AGE AFTER SUPPRESSION OF GROWTH BY DIETARY MEANS

Rat	Growth resumed at		Final maximum body weight (g)
	Age (days)	Body weight (g)	
1	552	170	204
2	537	108	187
3	512	58	222
4	479	167	228
5	401	104	259

the resumption of the growth function " In a later paper (1916) they clearly recognized that growth following this type of repression often proceeded at an enormously exaggerated rate Thus a female rat, on refeeding after maintenance for some time without growth at a weight of about 100 g, gained 112 g in 26 days Again, another showed a gain of 150 g in 36 days at a size which normally required more than 200 days for the same growth accomplishment

More recently Clemmensen (1933) retarded the growth of young rats by feeding them on a diet deficient in vitamin B The treatment began at 4 weeks of age and extended for various periods up to 78 weeks There was considerable mortality, but the survivors had apparently preserved their capacity for normal growth upon adequate refeeding, so that ultimately the body weight of the controls was equalled

Guastalla and Rigoletti (1935) similarly retarded the growth of rats by underfeeding them from 45 to 110 days of age Again there was high mortality, but the survivors upon subsequent refeeding grew rapidly and overtook the controls after 8 or 9 months

Jackson (1936) carried out extensive experiments on recovery in rats upon refeeding after prolonged suppression of growth by dietary deficiency of protein After being weaned at 3 weeks of age (when they weighed about 50 g) albino rats were maintained at nearly constant body weight for 15 weeks on a protein deficient diet On being re fed with the normal stock diet the surviving animals grew at first even more rapidly than the standard for corresponding body weight at about 9 months of age the female test rats had overtaken the normal controls, although the males still tended to lag somewhat behind

#### C—General Principles

The above experiments exemplify various fundamental responses which it is advantageous to describe and to classify in terms of rate of growth

Fig. 12, which is confined to reactions described or discussed in the present paper, is a partly schematic representation along these lines. It would

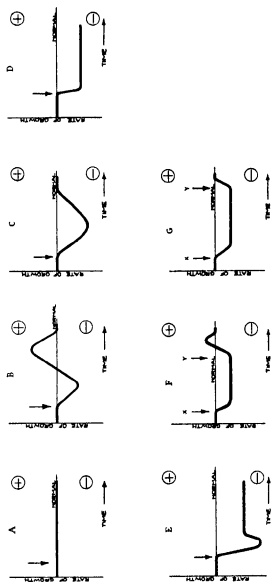


FIG. 12.—Examples of the influence of various agents on the rate of growth. A, after administration of some non-carcinogenic compounds, e.g. pyrene. B, response to moderate doses of certain toxic substances, e.g. colchicine. C, after larger doses of certain toxic substances. D, response to 1.25  $\mu$ -dibenzanthracene, 10 mg. E, after administration of a more toxic carcinogenic hydrocarbon such as 1.25  $\mu$ -dibenzanthracene, 10 mg. F and G, retardation and recovery after successive withdrawal (X) and supply (Y) of protein or vitamin B.

appear that increasing doses of certain substances, of which colchicine is a good example, produce in turn (a) no apparent response (after suitably small dosage), (b) a triphasic response (after medium doses) in which the growth

rate successively falls below the normal level compensates by rising above the normal and finally subsides to bring about total recovery and (c) a diphasic response (after larger doses) in which the growth rate is first depressed and then returns to normal without a period of increase above that level.

The outstanding feature in the experiments with X radiation lead or colchicine is the uniform tendency to recovery to the original growth rate after a single application or exposure even in cases where the latter resulted in signs or symptoms of general toxicity. On the other hand the response to a single injection of the carcinogenic hydrocarbons employed presents a striking difference. In the case of 1 2 5 6 dibenzanthracene the reaction is strictly monophasic taking the form of an abrupt fall in growth rate to a level which remains constant for long periods at least. The significance of this result is of course further increased by contrast with the inactivity of the non carcinogenic compounds tested under the same conditions. 1 2 5 6 dibenzacridine is of additional interest as appearing to possess greater inherent toxicity (as compared with 1 2 5 6 dibenzanthracene) so that the change in growth rate immediately after administration is complicated by a superimposed fall from which recovery soon takes place to the new subnormal level.

For comparative purposes fig. 12 also indicates (a) the fall in growth rate produced by continued deprivation of protein or vitamin and (b) the rapid recovery—with or without compensation—which follows the complete restoration of these substances to the diet.

We are indebted to the British Empire Cancer Campaign and to the Medical Research Council for expenses grants (to A. H. and C. M. S. respectively). It is also a pleasure to record our thanks to Professor J. W. Cook who supplied the samples of 1 2 5 6 dibenzacridine (chrysene and dodecahydro 1 2 benzantracene) to Professor A. J. Clark, F.R.S. for his interest in the work and to Dr. I. Hieger for his help in determining the rate of disappearance of 1 2 5 6 dibenzanthracene.

#### IV—SUMMARY

1. The carcinogenic compounds 1 2 5 6 dibenzanthracene, 1 2 5 6 dibenzacridine and 3 4 benzpyrene produced an immediate constant and long continued reduction in the rate of growth of young rats.

2. The non carcinogenic substances pyrene, fluoranthene and dodecahydro 1 2 benzantracene did not show this inhibitory influence on growth when tested under the same conditions.

3 Several agencies (X radiation, lead and colchicine) were found to produce a temporary interference which was followed by recovery to the normal growth rate with or without compensation Phenobarbitone (luminal) had no inhibitory action of any kind

4 These effects are compared and their significance discussed

# V—APPENDIX

The following tables show the mean and individual weights (in g) of animals at various periods of the foregoing experiments, including always the first day (start of preliminary weighings), the day on which the injection or other treatment was given, and another day later in the experiment

TABLE XIII

Treatment	Dose (mg)	No of animals	Deaths		
			Within 20 days of injection	Within 40 days of injection	Within 60 days of injection
1 2 5 6 dibenz anthracene	3	13	0	—	—
	10	13	0	0	3
	(Exp 1)				
	10	9	1	2	—
	(Exp 7)				
	30	12	0	0	—
	90	9	0	4	7
1 2 5 6 dibenz acridine	10	9	0	1	—
	30	10	1	5	—
3 4 benzpyrene	10	10	0	0	—
Chrysene	30	10	0	1	—
Control	0	115	0	3	4

Table XIII shows the deaths in animals treated with 1 2 5 6 dibenzanthracene, 1 2 5 6 dibenzacridine, 3 4 benzpyrene and chrysene, and their controls

## Exp 1 (male animals only)

Treatment on 8th day	Rat no	1st day	8th day	100th day
Control oil	1	57	66	220
	2	56	73	210
	3	54	64	200
	4	50	62	255
	5	78	91	265
	6	46	54	170
	7	54	71	220
	8	50	58	198
	9	71	93	260
	Mean	57.3	70.2	222.0

*Exp 1 (male animals only)—continued*

Treatment on 8th day	Rat no	1st day	8th day	100th day
10 mg 1 2 5 6 dibenzanthracene	10	57	73	136
	11	55	66	120
	12	46	63	138
	13	52	64	133
	14	50	62	166
	15	50	60	178
	16	65	84	180
	Mean	53.5	67.4	150.1
10 mg pyrene	17	58	72	208
	18	48	56	155
	19	48	58	260
	20	66	84	255
	21	64	78	260
	22	51	62	195
	23	46	58	192
	24	60	80	220
	Mean	55.1	68.5	218.1

*Exp 2 (male animals only)*

Treatment on 7th day	Rat no	1st day	7th day	38th day
Control untreated	1	43	54	97
	2	56	68	110
	3	54	64	110
	4	77	92	155
	5	72	86	137
	Mean	60.4	72.8	121.8
Control oil	6	79	97	150
	7	50	62	106
	8	81	101	156
	9	71	85	141
	10	60	75	142
	11	72	79	128
	Mean	68.8	83.1	137.1
30 mg 1 2 5 6 dibenzanthracene	12	47	61	108
	13	73	93	118
	14	55	61	78
	15	80	93	123
	16	73	86	116
	Mean	65.6	78.8	108.6



*Exp 3*

Treatment on 8th day	Rat no	1st day	8th day	14th day	26th day
Control oil	1	58	71	86	102
	2	68	81	95	119
	3	59	72	83	103
	4	81	91	108	127
	5	70	80	95	114
	6	63	77	87	105
	7	65	78	91	108
	8	49	57	71	84
	9	63	76	89	102
	Mean	64.0	75.9	89.4	107.1
90 mg 1 2 5 6 dibenzanthracene	10	61	69	—	81
	11	55	63	—	63
	12	73	87	—	99
	13	75	89	—	95
	14	62	76	—	79
	15	64	76	—	78
	16	64	79	—	91
	17	72	83	—	97
	18	62	71	—	71
	Mean	65.3	77.0	—	83.8
X irradiation (450 r)	19	55	65	73	—
	20	60	70	81	—
	21	80	106	114	—
	22	52	59	62	—
	23	82	94	99	—
	24	67	84	90	—
	25	71	88	92	—
	26	53	58	64	—
	27	73	85	92	—
	28	44	52	54	—
	Mean	63.7	76.1	82.1	—

*Exp 4 (male animals only)*

Treatment on 8th day	Rat no	1st day	8th day	34th day
Control oil	1	66	82	133
	2	40	48	80
	3	75	94	130
	4	54	74	119
	5	65	89	154
	6	66	84	123
	7	42	49	77
	Mean	58.3	74.3	116.5

*Exp 4 (male animals only)*—continued

Treatment on 8th day	Rat no	1st day	8th day	34th day
3 mg 1 2 5 6 dibenzanthracene	8	56	67	95
	9	73	97	145
	10	69	97	126
	11	57	74	115
	12	54	67	103
	13	62	83	122
	14	48	56	85
	Mean	59.8	77.3	113.0

*Exp 5*

Treatment on 8th day	Rat no	1st day	8th day	13th day	30th day
Control oil	1	90	109	125	166
	2	87	106	115	141
	3	113	126	131	144
	4	93	104	113	133
	5	65	80	87	97
	6	59	71	81	104
	7	53	66	75	102
	8	71	82	95	138
	9	62	76	83	106
	10	69	87	96	132
	Mean	76.2	90.7	100.1	126.3
30 mg chrysene	11	80	96	97	128
	12	73	84	87	103
	13	94	117	124	167
	14	73	83	83	102
	15	95	113	117	138
	16	60	71	71	77
	17	78	98	104	144
	18	62	78	83	104
	19	90	107	110	149
	20	58	73	76	107
	Mean	76.3	92.0	95.2	121.9
30 mg 1 2 5 6 dibenzacridine	21	67	72	65	57
	22	98	112	112	131
	23	80	86	69	†
	24	104	120	124	†
	25	81	99	100	109
	26	70	82	81	77
	27	60	74	73	99
	28	65	77	77	86
	29	67	79	77	99
	30	71	86	85	84
	Mean	76.3	88.7	86.3	92.7

*Exp. 6*

Treatment on 8th day	Rat no	1st day	8th day	24th day
Control oil	1	71	85	111
	2	39	42	53
	3	56	72	98
	4	62	72	100
	5	68	79	107
	6	81	91	†
	7	60	65	91
	8	82	95	131
	9	88	110	140
	10	87	101	146
	Mean 69.4		81.2	108.5
30 mg dodecahydro- 1 2-benzanthracene	11	78	93	126
	12	73	86	121
	13	55	72	96
	14	74	89	123
	15	67	82	118
	16	78	82	104
	17	75	91	128
	18	66	80	110
	Mean 70.7		84.4	115.7
30 mg fluoranthene	19	68	73	105
	20	76	94	125
	21	70	81	113
	22	79	93	134
	23	57	66	89
	24	59	65	105
	25	76	97	120
	26	72	90	124
	27	74	80	106
	Mean 70.1		82.1	113.4

*Exp. 7*

Treatment on 8th day	Rat no	1st day	8th day	10th day	30th day
Control oil	1	74	93	99	141
	2	80	95	101	Pregnant
	3	66	85	92	147
	4	65	67	61	96
	5	75	86	90	149
	6	60	73	75	112
	7	61	76	71	107
	8	53	62	63	94
	9	67	84	84	120
	Mean 66.7		80.1	81.8	120.7

*Exp 7—continued*

Treatment on 8th day	Rat no	1st day	8th day	10th day	30th day
10 mg 1 2 5 6 dibenzacridine	10	83	110	106	153
	11	68	85	83	103
	12	64	76	72	65
	13	64	75	69	93
	14	56	61	59	Sick
	15	60	72	66	83
	16	68	79	81	113
	17	50	60	59	66
	18	68	85	82	103
	Mean	64.5	78.1	75.2	97.4
10 mg 1 2 5 6 dibenzanthracene	19	70	75	77	92
	20	81	113	114	149
	21	63	78	75	89
	22	55	67	68	Sick
	23	68	73	69	87
	24	64	78	78	73
	25	50	60	59	†
	26	85	107	111	122
	Mean	67.0	81.3	81.3	102.0
10 mg 3 4 benz pyrene	27	77	100	99	130
	28	82	102	99	138
	29	74	96	94	129
	30	63	76	73	87
	31	65	74	73	91
	32	66	76	73	103
	33	61	73	69	101
	34	57	65	63	88
	35	59	68	62	84
	36	65	77	76	102
	Mean	66.9	80.7	78.1	105.3

*Exp 8*

Treatment on 15th day	Rat no	1st day	15th day	18th day	34th day
Control	1	67	87	90	110
	2	63	88	92	122
	3	45	55	55	72
	4	48	66	69	89
	5	55	80	82	116
	6	47	70	74	95
	7	55	82	88	117
	8	62	95	101	124
	9	59	91	95	125
	10	55	90	98	143
	11	59	102	112	147
	12	61	114	127	166
	13	62	107	117	158
	Mean	56.7	86.7	92.3	121.8

*Exp 8—continued*

Treatment on 15th day	Rat no	1st day	15th day	18th day	34th day
4 mg lead nitrate	14	41	55	57	69
	15	59	81	80	102
	16	78	105	106	134
	17	48	65	64	85
	18	59	80	78	105
	19	43	59	62	84
	20	44	64	66	90
	21	60	90	87	103
	22	65	93	100	135
	23	68	113	111	152
	24	58	97	98	128
	25	55	94	99	133
	26	60	103	101	142
	Mean	56.7	84.5	85.3	112.5

*Exp. 9*

Treatment on 22nd day	Rat no	1st day	22nd day	25th day	36th day
Control	1	57	93	101	127
	2	89	145	152	154
	3	74	127	134	Pregnant
	4	51	80	82	107
	5	64	103	110	133
	6	47	65	69	81
	7	67	108	112	127
	8	50	80	84	111
	9	67	124	129	162
	10	87	145	147	162
	11	70	101	102	142
	Mean	65.7	106.4	111.1	130.6
0.12 mg colchicine	12	74	125	125	151
	13	79	137	125	119
	14	52	74	†	†
	15	50	85	69	100
	16	65	113	†	†
	17	61	91	†	†
	18	45	74	72	89
	19	70	109	106	130
	20	77	121	118	142
	21	65	110	118	138
	22	69	97	80	105
	Mean	64.3	103.3	101.6	121.7

## Exp 9—continued

Treatment on 22nd day	Rat no	1st day	22nd day	25th day	36th day
10 mg phenobarbitone	23	78	—	—	162
	24	69	—	—	Pregnant
	25	46	—	—	114
	26	57	—	—	122
	27	53	—	—	129
	28	70	—	—	Pregnant
	29	68	—	—	143
	30	55	—	—	115
	31	73	—	—	149
	32	64	—	—	137
	33	80	—	—	159
	Mean	64.8	—	—	136.7

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